RECORD COPY
PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No. PCT/FI 9 8 / 0 0 7 4 9

International Filing Date 2 3 SEP 1998 (2 3. 09. 98)

The Finnish Patent Office
PCT International Application

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) 2971064PC/ko

Box No. I TITLE OF INVENTION

Novel gene

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

FINNISH IMMUNOTECHNOLOGY LTD
Pirkankatu 1 A 7
FIN-33210 Tampere
Finland

☐ This person is also inventor

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: FI

State *(that is, country)* of residence: FI

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

KROHN Kai
Iltarusko
Salmentaantie 751
FIN-36450 Salmentaka
Finland

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: FI

State *(that is, country)* of residence: FI

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

KOLSTER OY AB
Iso Roobertinkatu 23
P.O. Box 148
FIN-00121 Helsinki
Finland

Telephone No. 358-9-618821

Facsimile No. 358-9-602244

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

EL386267956US

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

HEINO Maarit
Mäkipäänkatu 27-29 C 74
FIN-33500 Tampere
Finland

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: FI

State *(that is, country)* of residence: FI

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

PETERSON Pärt
Kaskitie 13 F 61
FIN-33540 Tampere
Finland

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: EE

State *(that is, country)* of residence: FI

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SCOTT Hamish
43 Rue des Bains
CH-1205 Geneva
Switzerland

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: AU

State *(that is, country)* of residence: CH

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

ANTONARAKIS Stylianos
28 Boulevard des Philosophes
CH-1205 Geneva
Switzerland

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US & GR

State *(that is, country)* of residence: CH

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on another continuation sheet.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

KOLSTER OY AB
Iso Roobertinkatu 23
P.O. Box 148
FIN-00121 Helsinki
FINLANDE

Date of mailing (day/month/year)
26 May 1999 (26.05.99)

Applicant's or agent's file reference
2971064PC/ko

IMPORTANT NOTIFICATION

International application No.
PCT/FI98/00749

International filing date (day/month/year)
23 September 1998 (23.09.98)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
FINNISH IMMUNOTECHNOLOGY LTD.
Pirkankatu 1 A 7
FIN-33210 Tampere
Finland

State of Nationality: FI
State of Residence: FI
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
FINNISH IMMUNOTECHNOLOGY LTD.
Lenkkeilijänkatu 8
FIN-33520 Tampere
Finland

State of Nationality: FI
State of Residence: FI
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Eugénia Santos

Telephone No.: (41-22) 338.83.38

002635255

Form PCT/IB/306 (March 1994)

EL386267956US

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
KOLSTER OY AB
Iso Roobertinkatu 23
P.O. Box 148
FIN-00121 Helsinki
FINLANDE

09 -04- 1999

Date of mailing (day/month/year) 01 April 1999 (01.04.99)		
Applicant's or agent's file reference 2971064PC/ko	**IMPORTANT NOTICE**	
International application No. PCT/FI98/00749	**International filing date** (day/month/year) 23 September 1998 (23.09.98)	**Priority date** (day/month/year) 23 September 1997 (23.09.97)
Applicant FINNISH IMMUNOTECHNOLOGY LTD. et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,BR,CN,EP,IL,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AL,AM,AP,AT,AZ,BA,BB,BG,BY,CA,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   01 April 1999 (01.04.99) under No. WO 99/15559

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

2545927

Form PCT/IB/308 (July 1996)

EL386267956US

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

KOLSTER OY AB
Iso Roobertinkatu 23
P.O. Box 148
FIN-00121 Helsinki
FINLANDE

Date of mailing (day/month/year) 26 May 1999 (26.05.99)	
Applicant's or agent's file reference 2971064PC/ko	**IMPORTANT INFORMATION**
International application No. PCT/FI98/00749	**International filing date** (day/month/year) 23 September 1998 (23.09.98)
	Priority date (day/month/year) 23 September 1997 (23.09.97)
Applicant FINNISH IMMUNOTECHNOLOGY LTD. et al	

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,BR,CA,CN,CZ,DE,GB,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AL,AM,AT,AZ,BA,BB,BY,CH,CU,DK,EE,ES,FI,GE,GH,GM,HR,HU,ID,IS,KE,
   KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MW,MX,PT,SD,SG,SI,SL,TJ,TM,TR,TT,UA,
   UG,UZ,VN,YU,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

Eugénia Santos

Telephone No. (41-22) 338.83.38

2635251

Form PCT/IB/332 (September 1997)

EL386267956US

PCT / FI98 / 00749

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

LALIOTI Maria
C401
5 chemin Edouard Tavan
CH-1206 Geneva
Switzerland

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: CH

State *(that is, country)* of residence: CH

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SHIMIZU Nobuyoshi
4-1-W2103 Yukarigaoka
Sakura
Chiba
JP-285 Japan

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: JP

State *(that is, country)* of residence: JP

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

KUDOH Jun
6-14-8-303 Honkomagome
Bunkyo-ku
Tokyo
JP-113 Japan

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: JP

State *(that is, country)* of residence: JP

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

See Notes to the request form

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

☒ **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SZ** Swaziland, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)*

.....

National patent *(if other kind of protection or treatment desired, specify on dotted line)*:

☒ **AL** Albania .....
☒ **AM** Armenia .....
☒ **AT** Austria and utility model .....
☒ **AU** Australia .....
☒ **AZ** Azerbaijan
☒ **BA** Bosnia and Herzegovina .....
☒ **BB** Barbados
☒ **BG** Bulgaria .....
☒ **BR** Brazil .....
☒ **BY** Belarus
☒ **CA** Canada
☒ **CH and LI** Switzerland and Liechtenstein
☒ **CN** China .....
☒ **CU** Cuba .....
☒ **CZ** Czech Republic and utility model .....
☒ **DE** Germany and utility model .....
☒ **DK** Denmark and utility model .....
☒ **EE** Estonia and utility model .....
☒ **ES** Spain .....
☒ **FI** Finland and utility model .....
☒ **GB** United Kingdom
☒ **GE** Georgia .....
☒ **GH** Ghana .....
☒ **GM** Gambia
☒ **HR** Croatia
☒ **HU** Hungary .....
☒ **ID** Indonesia
☒ **IL** Israel .....
☒ **IS** Iceland
☒ **JP** Japan .....
☒ **KE** Kenya .....
☒ **KG** Kyrgyzstan .....
☒ **KP** Democratic People's Republic of Korea .....
.....
☒ **KR** Republic of Korea .....
☒ **KZ** Kazakstan .....
☒ **LC** Saint Lucia
☒ **LK** Sri Lanka
☒ **LR** Liberia
☒ **LS** Lesotho .....
☒ **LT** Lithuania
☒ **LU** Luxembourg .....
☒ **LV** Latvia
☒ **MD** Republic of Moldova .....
☒ **MG** Madagascar .....
☒ **MK** The former Yugoslav Republic of Macedonia
☒ **MN** Mongolia
☒ **MW** Malawi .....
☒ **MX** Mexico .....
☒ **NO** Norway
☒ **NZ** New Zealand .....
☒ **PL** Poland .....
☒ **PT** Portugal .....
☒ **RO** Romania
☒ **RU** Russian Federation .....
☒ **SD** Sudan
☒ **SE** Sweden
☒ **SG** Singapore
☒ **SI** Slovenia .....
☒ **SK** Slovakia and utility model .....
☒ **SL** Sierra Leone
☒ **TJ** Tajikistan .....
☒ **TM** Turkmenistan .....
☒ **TR** Turkey .....
☒ **TT** Trinidad and Tobago .....
☒ **UA** Ukraine .....
☒ **UG** Uganda .....
☒ **US** United States of America .....
☒ **UZ** Uzbekistan .....
☒ **VN** Viet Nam .....
☒ **YU** Yugoslavia .....
☒ **ZW** Zimbabwe .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet

☒ **GD** Grenada .....
☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box

	Filing Date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application receiving Office
item (1)	23 September 1997 (23.09.1997)	973762	FI		
item (2)					
item (3)					

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s) : (1)

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*
ISA /SE

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*
Date *(day/month/year)*: Number Country *(or regional Office)*

Box No. VIII CHECK LIST

This international application contains the following **number of sheets:**

request	: 5
description (excluding sequence listing part)	: 20
claims	: 3
abstract	: 1
drawings	: 9
sequence listing part of description	: 21
Total number of sheets	**: 59**

This international application is **accompanied by** the item(s) marked below

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language)
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other (specify): official action

Figure of the drawings which should accompany the abstract: -

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

KOLSTER OY AB

Leo Lehtonen

For receiving Office use only

1. Date of actual receipt of the purported international application:	23 SEP 1998 (23-09-1998)
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority specified by the applicant: ISA/ SE	6. ☐ Transmittal of search copy delayed until search fee is paid

2. Drawings: ☐ received ☐ not received:

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/47, A61K 38/17

A1

(11) International Publication Number: **WO 99/15559**

(43) International Publication Date: 1 April 1999 (01.04.99)

(21) International Application Number: PCT/FI98/00749

(22) International Filing Date: 23 September 1998 (23.09.98)

(30) Priority Data:
973762 23 September 1997 (23.09.97) FI

(71) Applicant *(for all designated States except US)*: FINNISH IMMUNOTECHNOLOGY LTD. [FI/FI]; Pirkankatu 1 A 7, FIN-33210 Tampere (FI).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KROHN, Kai [FI/FI]; Iltarusko, Salmentaantie 751, FIN-36450 Salmentaka (FI). HEINO, Maarit [FI/FI]; Mäkipäänkatu 27-29 C 74, FIN-33500 Tampere (FI). PETERSON, Pärt [EE/FI]; Kaskitie 13 F 61, FIN-33540 Tampere (FI). SCOTT, Hamish [AU/CH]; 43, rue des Bains, CH-1205 Geneva (CH). ANTONARAKIS, Stylianos [US/CH]; 28, boulevard des Philosophes, CH-1205 Geneva (CH). LALIOTI, Maria [CH/CH]; C401, 5, chemin Edouard Tavan, CH-1206 Geneva (CH). SHIMIZU, Nobuyoshi [JP/JP]; 4-1-W2103 Yukarigaoka, Sakura, Chiba 285 (JP). KUDOH, Jun [JP/JP]; 6-14-8-803 Honkomagome, Bunkyo-ku, Tokyo 113 (JP).

(74) Agent: KOLSTER OY AB; Iso Roobertinkatu 23, P.O. Box 148, FIN-00121 Helsinki (FI).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) **Title:** NOVEL GENE DEFECTIVE IN APECED AND ITS USE

(57) **Abstract**

The present invention relates to a novel gene, a novel protein encoded by said gene, a mutated form of the gene and to diagnostic and therapeutic uses of the gene or a mutated form thereof. More specifically, the present invention relates to a novel gene defective in autoimmune polyendocrinopathy syndrome type I (APS I), also called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (MIM No. 240,300).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00749

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/47, A61K 38/17
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAPLUS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Nature genetics, Volume 17, December 1997, The Finnish-German APECED Consortium, "An autoimmune disease, APECED, caused by mutations in a novel gene featuring two OHD-type zinc-finger domains" page 399 - page 403 --	1-8,19-24
A	Am. J. Hum. Genet., Volume 59, 1996, P. Björses et al, "Genetic Homogeneity of Autoimmune Polyglandular Disease Type I" page 879 - page 886 --	1-8,9-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 January 1999	25 -01- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerlöf Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

EL386267956US

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00749

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Genome Research, Volume 7, August 1997, Johanna Aaltonen et al, "High-Resolution Physical and Transcriptional Mapping of the Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy Locus on Chromosome 21q22.3 by FISH" page 820 - page 829	1-8,19-24

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00749

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 9-18
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Copy for the Elected Office (EO/US)

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

KOLSTER OY AB
Iso Roobertinkatu 23
P.O. Box 148
FIN-00121 Helsinki
FINLANDE

Date of mailing (day/month/year)
26 May 1999 (26.05.99)

Applicant's or agent's file reference
2971064PC/ko

IMPORTANT NOTIFICATION

International application No.
PCT/FI98/00749

International filing date (day/month/year)
23 September 1998 (23.09.98)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
FINNISH IMMUNOTECHNOLOGY LTD.
Pirkankatu 1 A 7
FIN-33210 Tampere
Finland

State of Nationality: FI
State of Residence: FI
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
FINNISH IMMUNOTECHNOLOGY LTD.
Lenkkeilijänkatu 8
FIN-33520 Tampere
Finland

State of Nationality: FI
State of Residence: FI
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Eugénia Santos
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 26 May 1999 (26.05.99)	
International application No. PCT/FI98/00749	**Applicant's or agent's file reference** 2971064PC/ko
International filing date (day/month/year) 23 September 1998 (23.09.98)	**Priority date** (day/month/year) 23 September 1997 (23.09.97)
Applicant KROHN, Kai et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   15 April 1999 (15.04.99)

   [ ] in a notice effecting later election filed with the International Bureau on:

   _____

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Eugénia Santos
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

2635249

Copy for the Elected Office (EO/US)

PCT/FI98/00749

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

KOLSTER OY AB
Iso Roobertinkatu 23
P.O. Box 148
FIN-00121 Helsinki
FINLANDE

Date of mailing (day/month/year)
13 March 2000 (13.03.00)

Applicant's or agent's file reference
2971064PC/ko

IMPORTANT NOTIFICATION

International application No.
PCT/FI98/00749

International filing date (day/month/year)
23 September 1998 (23.09.98)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
LALIOTI, Maria
C401
5, chemin Edouard Tavan
CH-1206 Geneva
Switzerland

State of Nationality: CH
State of Residence: CH
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [ ] the address [X] the nationality [ ] the residence

Name and Address
LALIOTI, Maria
C401
5, chemin Edouard Tavan
CH-1206 Geneva
Switzerland

State of Nationality: GR
State of Residence: CH
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Eugénia Santos

Telephone No.: (41-22) 338.83.38

17

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2971064PC/su		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/FI98/00749	23/09/1998	23/09/1997
International Patent Classification (IPC) or national classification and IPC		
C07K14/47		
Applicant		
FINNISH IMMUNOTECHNOLOGY LTD. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/04/1999	14.01.00
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Chavanne, F Telephone No. +49 89 2399 8399

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/FI98/00749

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-41 as originally filed

Claims, No.:

1-24 with telefax of 11/10/1999

Drawings, sheets:

1/10-10/10 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 23.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 23 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-22, 24
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-22, 24
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-8, 21, 24
	No:	Claims	9-20, 22

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Claim 23 lacks clarity due to the expression "reagents". This expression is not suitable to define the scope of the claim, because there is no technical characterisation of said compound. Moreover, it may encompass almost all possible compounds, and possibly also known compounds. Therefore, an evaluation with regard to novelty and inventive step cannot be carried out.

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The examination of the present application has been performed assuming that the claimed priority is valid. However, it is noted that an intermediate document would then become relevant to assess the patentability of any claimed subject-matter not entitled to said priority.

2. The isolated DNA sequence consisting of the SEQ. ID. No: 1 and the corresponding protein sequence of amino acid sequence SEQ ID No: 2 are new and inventive over the prior art. Thus, claims 1-22 and 24 may be considered new and inventive.

VI. Certain documents cited

Certain published documents (Rule 70.10)

1. Nature Genetics
   Vol. 17, pp. 399-403, 1997

VII. Certain defects in the international application

1. Apart from a vague reference to SEQ ID No. 1 and 2 (page 3, lines 28 and 35), there are no references to said SEQ. IDs in the present application, which would allow a clear characterisation of said sequences. It appears necessary to make references to SEQ. ID No. 1 and 2 when the nucleotide and the amino acid sequences, respectively, of the AIR-1 product are mentioned.

VIII. Certain observations on the international application

1. The formulations "An isolated DNA sequence comprising the sequence...", "A DNA sequence having the sequence...", "A protein comprising the amino acid sequence..." or "A protein having the amino acid sequence..." in claims 1, 4, 5, 7, 9 and 16 do not clearly define the scope of the claims in that the length of said sequences has no limitation. Thus, the expressions "comprising" and "having" should be replaced with "consisting of" (Article 6 PCT).

2. Claims 1 and 9 lack clarity in that the formulation "a functionnally equivalent" is not clearly defined, and thus, opened to interpretation (Article 6 PCT). Moreover, the man skilled in the art, faced with the problem of isolating such a functionnally equivalent DNA sequence would not be able to perform it without intensive experimentations of undue burden.

3. For the assessment of the present claims 9-20 and 22 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Claims

1. An isolated DNA sequence characterized by comprising the sequence id. no. 1 or a functional fragment or variant thereof, or an functionally equivalent isolated DNA sequence hybridizable thereto.

2. An isolated DNA sequence according to claim 1, characterized in that it is associated with diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

3. An isolated DNA-sequence according to claim 1 or 2, characterized in that it includes a gene defect responsible for APECED.

4. A DNA sequence according to claim 1, characterized by having the sequence according to sequence id. no 1 or a functional fragment thereof having the sequence according to sequence id. no 3 or sequence id. no 5.

5. A protein characterized by comprising the amino acid sequence id. no. 2 or a functional fragment or variant thereof.

6. A protein according to claim 5, characterized in that it is associated diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

7. A protein according to claim 5 or 6, characterized by having the amino acid sequence id. no. 2, or a functional fragment thereof having the sequence according to sequence id. no. 4, or a functional fragment thereof having the sequence according to sequence id. no 6.

8. A protein according to any of claims 5 to 7, characterized by having distinct structural motifs, including the PHD finger motif (PHD), the LXXLL motif (L), proline-rich region (PRR), and cystein-rich region (CRR).

9. A method for the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, characterized by detecting in a biological specimen the presence of a DNA sequence comprising the sequence id. no. 1 or a functional fragment or variant thereof, or a functionally equivalent isolated DNA-sequence hybridizable thereto.

10. A method according to claim 9, characterized in that the DNA sequence is associated with APECED.

11. A method according to claim 9 or 10, characterized in that the DNA sequence includes a gene defect responsible for APECED.

12. A method according to claim 11, characterized in that the gene defect to be detected includes a "C" to "T" transition resulting in the "Arg" to "Stop" nonsense mutation at amino acid position 257 and/or a "A" to "G" transversion resulting in the "Lys" to "Glu" missense mutation at amino acid position 42.

13. A method according to any one of claims 9 to 12, characterized in that DNA techniques are used for the detection.

14. A method according to any one of claims 9 to 13, characterized in that the detection takes advantage of TaqI or another enzyme cleaving at recognition site 5'-TCGA-3' digestion.

15. A method according to any one of claims 9 to 14, characterized in that the disease is autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

16. A method for the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, characterized by detecting in a biological specimen the presence or the absence of a protein comprising the sequence id. no. 1, or a functional fragment thereof having the sequence according to sequence id. no. 4, or a functional fragment thereof having the sequence according to sequence id. no. 6.

17. A method according to claim 16, characterized in that the protein is associated with APECED.

18. A method according to claim 16 or 17, characterized in that the disease is autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

19. The use of the DNA sequence according to any one of claims 1 to 4 in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

20. The use of the protein according to any one of claims 5 to 7 in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

21. The use of the DNA sequence according to any one of claims 1 to 4 for the preparation of a medicament useful in a gene therapy method of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

22. The use of the DNA sequence according to any one of claims 1 to 4 in the treatment of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

23. Reagents reacting with the DNA sequence according to any one of claims 1 to 4 or the protein of any one of the claims 5 to 8 or with reagents reacting therewith.

24. Reagents according to claim 23, characterized in that they are antibodies.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2971064PC/su		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/FI98/00749	23/09/1998	23/09/1997
International Patent Classification (IPC) or national classification and IPC		
C07K14/47		
Applicant		
FINNISH IMMUNOTECHNOLOGY LTD. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/04/1999	14.01.00
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Chavanne, F Telephone No. +49 89 2399 8399

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/FI98/00749

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-41 as originally filed

Claims, No.:

1-24 with telefax of 11/10/1999

Drawings, sheets:

1/10-10/10 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 23.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 23 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-22, 24
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-22, 24
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-8, 21, 24
	No:	Claims	9-20, 22

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Claim 23 lacks clarity due to the expression "reagents". This expression is not suitable to define the scope of the claim, because there is no technical characterisation of said compound. Moreover, it may encompass almost all possible compounds, and possibly also known compounds. Therefore, an evaluation with regard to novelty and inventive step cannot be carried out.

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The examination of the present application has been performed assuming that the claimed priority is valid. However, it is noted that an intermediate document would then become relevant to assess the patentability of any claimed subject-matter not entitled to said priority.

2. The isolated DNA sequence consisting of the SEQ. ID. No: 1 and the corresponding protein sequence of amino acid sequence SEQ ID No: 2 are new and inventive over the prior art. Thus, claims 1-22 and 24 may be considered new and inventive.

VI. Certain documents cited

Certain published documents (Rule 70.10)

1. Nature Genetics
   Vol. 17, pp. 399-403, 1997

VII. Certain defects in the international application

1. Apart from a vague reference to SEQ ID No. 1 and 2 (page 3, lines 28 and 35), there are no references to said SEQ. IDs in the present application, which would allow a clear characterisation of said sequences. It appears necessary to make references to SEQ. ID No. 1 and 2 when the nucleotide and the amino acid sequences, respectively, of the AIR-1 product are mentioned.

VIII. Certain observations on the international application

1. The formulations "An isolated DNA sequence comprising the sequence...", "A DNA sequence having the sequence...", "A protein comprising the amino acid sequence..." or "A protein having the amino acid sequence..." in claims 1, 4, 5, 7, 9 and 16 do not clearly define the scope of the claims in that the length of said sequences has no limitation. Thus, the expressions "comprising" and "having" should be replaced with "consisting of" (Article 6 PCT).

2. Claims 1 and 9 lack clarity in that the formulation "a functionnally equivalent" is not clearly defined, and thus, opened to interpretation (Article 6 PCT). Moreover, the man skilled in the art, faced with the problem of isolating such a functionnally equivalent DNA sequence would not be able to perform it without intensive experimentations of undue burden.

3. For the assessment of the present claims 9-20 and 22 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Claims

1. An isolated DNA sequence characterized by comprising the sequence id. no. 1 or a functional fragment or variant thereof encoding a protein having the same functional activity, or an functionally equivalent isolated DNA sequence hybridizable thereto.

2. An isolated DNA sequence according to claim 1, characterized in that it is associated with diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

3. An isolated DNA-sequence according to claim 1 or 2, characterized in that it includes a gene defect responsible for APECED.

4. A DNA sequence according to claim 1, characterized by having the sequence according to sequence id. no 1 or a functional fragment thereof having the sequence according to sequence id. no 3 or sequence id. no 5.

5. A protein characterized by comprising the amino acid sequence id. no. 2 or a functional fragment or variant thereof having the same functional properties.

6. A protein according to claim 5, characterized in that it is associated diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

7. A protein according to claim 5 or 6, characterized by having the amino acid sequence id. no. 2, or a functional fragment thereof having the sequence according to sequence id. no. 4, or a functional fragment thereof having the sequence according to sequence id. no 6.

8. A protein according to any of claims 5 to 7, characterized by having distinct structural motifs, including the PHD finger motif (PHD), the LXXLL motif (L), proline-rich region (PRR), and cystein-rich region (CRR).

9. A method for the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, characterized by detecting in a biological specimen the presence of a DNA sequence comprising the sequence id. no. 1 or a functional fragment or variant thereof encoding a protein having the same functional activity, or a functionally equivalent isolated DNA-sequence hybridizable thereto.

10. A method according to claim 9, characterized in that the DNA sequence is associated with APECED.

11. A method according to claim 9 or 10, characterized in that the DNA sequence includes a gene defect responsible for APECED.

12. A method according to claim 11, characterized in that the gene defect to be detected includes a "C" to "T" transition resulting in the "Arg" to "Stop" nonsense mutation at amino acid position 257 and/or a "A" to "G" transversion resulting in the "Lys" to "Glu" missense mutation at amino acid position 42.

13. A method according to any one of claims 9 to 12, characterized in that DNA techniques are used for the detection.

14. A method according to any one of claims 9 to 13, characterized in that the detection takes advantage of TaqI or another enzyme cleaving at recognition site 5'-TCGA-3' digestion.

15. A method according to any one of claims 9 to 14, characterized in that the disease is autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

16. A method for the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, characterized by detecting in a biological specimen the presence or the absence of a protein comprising the sequence id. no. 2, or a functional fragment thereof having the sequence according to sequence id. no. 4, or a functional fragment thereof having the sequence according to sequence id. no. 6.

17. A method according to claim 16, characterized in that the protein is associated with APECED.

18. A method according to claim 16 or 17, characterized in that the disease is autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

19. The use of the DNA sequence according to any one of claims 1 to 4 in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

20. The use of the protein according to any one of claims 5 to 7 in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune poly-endocrinopathy-candidiasis-ectodermal dystrophy (APECED).

21. The use of the DNA sequence according to any one of claims 1 to 4 for the preparation of a medicament useful in a gene therapy method of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

22. The use of the DNA sequence according to any one of claims 1 to 4 in the treatment of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

23. Reagents reacting with the DNA sequence according to any one of claims 1 to 4 or the protein of any one of the claims 5 to 8 or with reagents reacting therewith.

24. Reagents according to claim 23, characterized in that they are antibodies.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2971064PC/su		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/FI98/00749	23/09/1998	23/09/1997
International Patent Classification (IPC) or national classification and IPC C07K14/47		
Applicant FINNISH IMMUNOTECHNOLOGY LTD. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/04/1999	14.01.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Chavanne, F Telephone No. +49 89 2399 8399

Form PCT/IPEA/409 (cover sheet) (January 1994)

EL386267956US

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/FI98/00749

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-41 as originally filed

Claims, No.:

1-24 with telefax of 11/10/1999

Drawings, sheets:

1/10-10/10 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 23.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 23 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-22, 24
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-22, 24
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-8, 21, 24
	No:	Claims	9-20, 22

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Claim 23 lacks clarity due to the expression "reagents". This expression is not suitable to define the scope of the claim, because there is no technical characterisation of said compound. Moreover, it may encompass almost all possible compounds, and possibly also known compounds. Therefore, an evaluation with regard to novelty and inventive step cannot be carried out.

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Certain published documents (Rule 70.10)

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   Vol. 17, pp. 399-403, 1997

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1. The formulations "An isolated DNA sequence comprising the sequence...", "A DNA sequence having the sequence...", "A protein comprising the amino acid sequence..." or "A protein having the amino acid sequence..." in claims 1, 4, 5, 7, 9 and 16 do not clearly define the scope of the claims in that the length of said sequences has no limitation. Thus, the expressions "comprising" and "having" should be replaced with "consisting of" (Article 6 PCT).

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3. For the assessment of the present claims 9-20 and 22 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Claims

1. An isolated DNA sequence characterized by comprising the sequence id. no. 1 or a functional fragment or variant thereof encoding a protein having the same functional activity, or an functionally equivalent isolated DNA sequence hybridizable thereto.

2. An isolated DNA sequence according to claim 1, characterized in that it is associated with diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

3. An isolated DNA-sequence according to claim 1 or 2, characterized in that it includes a gene defect responsible for APECED.

4. A DNA sequence according to claim 1, characterized by having the sequence according to sequence id. no 1 or a functional fragment thereof having the sequence according to sequence id. no 3 or sequence id. no 5.

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17. A method according to claim 16, characterized in that the protein is associated with APECED.

18. A method according to claim 16 or 17, characterized in that the disease is autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

19. The use of the DNA sequence according to any one of claims 1 to 4 in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

20. The use of the protein according to any one of claims 5 to 7 in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune poly-endocrinopathy-candidiasis-ectodermal dystrophy (APECED).

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22. The use of the DNA sequence according to any one of claims 1 to 4 in the treatment of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

23. Reagents reacting with the DNA sequence according to any one of claims 1 to 4 or the protein of any one of the claims 5 to 8 or with reagents reacting therewith.

24. Reagents according to claim 23, characterized in that they are antibodies.

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(54) Title: NOVEL GENE DEFECTIVE IN APECED AND ITS USE

(57) Abstract

The present invention relates to a novel gene, a novel protein encoded by said gene, a mutated form of the gene and to diagnostic and therapeutic uses of the gene or a mutated form thereof. More specifically, the present invention relates to a novel gene defective in autoimmune polyendocrinopathy syndrome type I (APS I), also called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (MIM No. 240,300).

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NOVEL GENE DEFECTIVE IN APECED AND ITS USE

Field of the invention

The present invention relates to a novel gene, a novel protein encoded by said gene, a mutated form of the gene and to diagnostic and therapeutic uses of the gene or a mutated form thereof. More specifically, the present invention relates to a novel gene defective in autoimmune polyendocrinopathy syndrome type I (APS I), also called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (MIM No. 240,300).

Background

Autoimmune polyglandular syndrome type I (APS I), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), is a rare recessively inherited disease (MIM No. 240,300) that is more prevalent among certain isolated populations, such as Finnish, Sardinian and Iranian Jewish populations. The incidence of the disease among the Finns and the Iranian Jews is estimated to be 1:25000 and 1:9000, respectively, whereas only few cases in other parts of the world are found each year.

APECED is one of the two major autoimmune polyendocrinopathy syndromes. The causing factor of APECED has not yet been identified. The syndrome is characterized by lack of tolerance to numerous self-antigens and can therefore be considered as a prototype of organ-specific autoimmune diseases. In APECED, the patient develops chronic mucocutaneous candidiasis soon after birth, and later several organ-specific autoimmune diseases, mainly hypoparathyreoidism, Addison's disease, chronic atrophic gastritis with or without pernicious anemia, and in puberty gonadal dysfunction occur [Ahonen P, Clin. Genet. 27 (1985) 535-542]. An accepted criterion for diagnosis of APECED is the presence of at least two of the three main symptoms, Addison's disease, hypoparathyroidism and candidiasis, in patients [Neufeld, M. *et al.*, Medicine 60 (1981) 355-362]. Immunologically, the major findings are the presence of high-titer serum autoantibodies against the effected organs, antibodies against *Candida albicans*, and low or lacking T-cell responses toward candidal antigens [Blizzard, R. M. and Kyle M., J. Clin. Invest. 42 (1963) 1653-1660; Arulanantham, K. *et al.*, New Eng. J. Med. 300 (1979) 164-168; Krohn, K. *et al.*, Lancet 339 (1992) 770-773; Uibo R. *et al.*, J. Clin. Endocrinol. Metab. 78 (1994) 323-328]. The disease usually occurs in

childhood, but new tissue specific symptoms may appear throughout life [Ahonen, P. *et al.*, New Engl. J. Med. 322 (1990) 1829-1836]. APECED is not associated with a particular HLA haplotype, and both males and females are equally affected consistant with the autosomal recessive mode of inheritance.

The locus for the APECED gene has been mapped to chromosome 21q22.3 between gene markers D21S49 and D21S171 based on linkage analysis of Finnish families [Aaltonen, J. *et al.*, Nature Genet. 8 (1994) 83-87]. Recently, Börses *et al.* reported a maximum LOD score of 10.23 with marker D21S1912 just proximal to the gene PFKL, and thus by linkage disequilibrium studies the critical region for APECED can be considered to be less than 500 kb between markers D21S1912 and D21S171. Locus heterogeneity was not revealed by linkage analysis of non-Finnish families [Björses, P. *et al.*, Am. J. Hum. Genet. 59 (1996) 879-886].

For the APECED gene, the name "autoimmune regulator" or "AIRE" has been adopted by the scientific community after the priority date of the present application. Similarly the protein encoded by the AIRE gene is now called the "AIRE protein".

Physical maps of human chromosome 21q22.3 have been developed using YACs, and bacterial based large insert cloning vectors [Chumakov *et al.*, Nature 359 (1992) 380; Stone et al., Genome Res. 6 (1996) 218], and many laboratories have contributed to the construction of a transcription map of the whole chromosome and 21q22.3 in particular [Chen *et al.*, Genome Res. 6 (1996) 747-760; Yaspo *et al.*, Hum. Mol. Genet. 4 (1995) 1291-1304]. Numerous trapped exons from chromosome 21 specific cosmids and also physical contigs from the APECED critical region have been identified and partially characterized. In addition, a number of ESTs from the international human genome project have been mapped to the APECED critical region.

Recently, as part of the international efforts of generating the entire sequence of human chromosome 21 and international agreements on the immediate availability of this type of sequence data, the partial sequence of the APECED gene critical region was made available in GenBank by the Stanford Human Genome Center which is currently carrying out the sequencing of 1.0 Mb around the critical region of the APECED gene.

However, the precise location and the sequence of the APECED gene and the nature of the gene product have not so far been clarified. Thus

at present the diagnosis of APECED is based mainly on developed clinical symptoms and typical clinical findings, e.g. the presence of autoantibodies against adrenal cortex or steroidogenic enzymes P450c17 and/or P450scc. The linkage analysis is seldom used. Further, means for natal or presymptomatic diagnosis of the disease are not easily available, since the linkage analysis provides only an indirect data through known gene markers and requires samples from several family members in several generations. Additionally, the linkage analysis is tedious and can be performed only in specialized laboratories by highly-skilled personnel.

Also the mapping of the carriers of the disease gene is presently based on the linkage analysis and thus not readily available.

Summary of the invention

We have now identified a novel gene encoding a novel zinc finger protein, designated as autoimmune regulator 1 or AIR-1, which is mutated in APECED. The novel gene and protein allow further development of the diagnosis and therapy of diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED.

The object of the invention is to provide means which are useful in a diagnostic method and a gene therapeutic method in the diagnosis and treatment of diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED.

Another object of the invention is to provide a novel method for the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED, including the pre- and postnatal diagnosis and the mapping of the carriers, the method being easy and reliable to perform.

The present invention relates to an isolated DNA sequence comprising the sequence id. no. 1 or a functional fragment or variant thereof, or a functionally equivalent isolated DNA sequence hybridizable thereto, the DNA sequence being associated with diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED. Preferably said isolated DNA sequence includes a gene defect responsible for APECED.

The present invention also relates to a protein comprising the amino acid sequence id. no. 2 or a functionally equivalent fragment or variant thereof, the protein being associated with diseases related to immune maturation and

regulation of immune response towards self and nonself, such as APECED. Said protein has distinct structural motifs, including the PHD finger motif (PHD), the LXXLL motif (L), proline-rich region (PRR), and cystein-rich region (CRR).

The present invention further relates to a method for the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED, comprising detecting in a biological specimen the presence of a DNA sequence comprising the sequence id. no. 1 or a functional fragment or variant thereof, or a functionally equivalent DNA-sequence hybridizable thereto, the DNA sequence being associated with diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED.

The present invention further relates to the use of the above-identified DNA-sequences in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED.

The present invention further relates to a method for the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED, comprising detecting in a biological specimen the presence or the absence of a protein comprising the sequence id. no. 2 or a functionally equivalent fragment thereof, the protein being associated with diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED.

The present invention further relates to the use of the above-identified protein or a functionally equivalent fragment thereof in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED.

The present invention further relates to the use of the above-identified DNA sequences in gene therapy or for the preparation of a pharmaceutical preparation useful in a gene therapy method of diseases related to immune maturation and regulation of immune response towards self and nonself, such as APECED.

Brief description of the drawings

Figure 1 shows a physical map of the APECED gene locus in the chromosome 21q22.3. Cosmids D1G8, D40G11, D9G11, D28B11, and D4G11, overlapping clones used for the genomic sequencing [Kudoh, J. *et al.*,

DNA Res. 4 (1997) 45 -52] are indicated by horizontal lines. The APECED gene located just proximal to the 5' end of the neighboring gene PFKL is indicated by a solid arrow. N indicates *Not*I sites. DNA marker D21S1912 is shown as open box.

Figure 2 shows the structures of the APECED gene and AIR proteins. (A) Cloning strategy of APECED cDNAs and the order of the exons in the APECED gene. DNA fragments amplified by PCR and 3'- and 5'-RACE are indicated by the lines. Exon 1' is the 5'-noncoding exon of the AIR-2 and AIR-3. An additional alternative splicing of AIR-3 in exon 10, resulting in an amino acid change in its downstream, is indicated by vertical lines. Each exon, except exon 1', is bordered by the common splice site consensus sequence, ag:gt. Mutations in the exon 2 and exon 6 are indicated by the arrows. (B) Schematic presentation of the three AIR proteins showing distinct structural motifs, including the PHD finger motif (PHD), the LXXLL motif (L), proline-rich region (PRR), and cystein-rich region (CRR).

Figure 3 shows electropherograms showing the sequence surrounding the mutations in the APECED gene. (A) Mutation analysis of a Swiss APECED family. The parents are heterozygous for the allele (normal "C" and abnormal "T"). The affected boy and girl show the "C" to "T" transition resulting in the "Arg" to "Stop" nonsense mutation at amino acid position 257. (B) Mutation analysis of two Finnish APECED patients. The patient MP is homozygous for the mutant allele (left), NP is heterozygous for the allele (right). (C) The patient NP shows the "A" to "G" transversion resulting in the "Lys" to "Glu" missense mutation at amino acid position 42. FLEB is a normal control.

Figure 4 shows the result of a restriction enzyme *Taq*I digestion assay demonstrating the R257stop mutation. Four APECED patients [HP1 (lane 1), HP2 (lane 2), NP (lane 6), and MP (lane 8)], the mothers of two families [HM (lane 5) and NM (lane 7)], two healthy siblings [HN1 (lane 3) and HN2 (lane 4)] of family H and normal controls [C1, C2 and C3 (lanes 9-11)] are shown. The APECED patients HP1, HP2 and MP are homozygotes for the R257stop mutation. The APECED patient NP is heterozygous for the R257stop mutation but is carrying a mutation at a different position in another allele of the APECED gene (shown above in Fig. 3C). Both mothers (HM and NM) and two healthy siblings (HN1 and HN2) are heterozygous for the R257stop mutation and therefore carriers of APECED but are not having the

disease. Two controls (C1 and C2) are both homozygous for normal alleles. Normal alleles produce a lower 225 bp fragment, the mutated fragment is upper band at 285 bp.

Figure 5 shows an amino acid sequence alignment for the PHD finger motif of AIR-1, Mi-2, and TIF1. The consensus amino acid residues conserved in the PHD finger motif is indicated by the bold letters underneath. The residues that are identical with AIR-1 (aa 299-340) are shown by the dots. GenBank accession nos. of Mi-2 and TIF1 are X86691 and AF009353, respectively.

Figure 6 is a Western blot showing the expression of AIR-1 in fetal liver. A sample of fetal liver was run on PAGE, transferred to a nitrocellulose filter and probed with sera as follows: Lane 1, control mouse serum, lane 2, control mouse serum absorbed with peptide AIR-1/2 (sequence id. no. 25), lanes 3 and 4, serum from a mouse immunized with peptide AIR-1/2 for four and six weeks, respectively and absorbed with peptide AIR-1/2, lanes 5 and 6, unabsorbed serum from a mouse immunized with peptide AIR-1/2 for four and six weeks, respectively. The strong band seen in lanes 5 and 6 represent the AIR-1 protein with a molecular weight of approx. 58 kD, the lower band is an approx. 20 kD breakdown product of the AIR protein. The bands seen in all lanes are non-specific.

Figure 7 shows the expression of the APECED mRNA (7A) or the AIR protein (7B, 7C and 7D) demonstrated by in situ hybridization (7A) or by immunohistochemistry (7B, 7C and 7D). Figure 7A shows APECED mRNA positive cells scattered in the medullary region of human thymus. Figure 7B shows similar cells with the same localization now stained for the AIR protein. Figure 7C is a higher magnification of 7B, showing the localization of the AIR protein in the nuclei. Note the speckled localiation pattern in the nuclei. Figure 7D shows the cytoplasmic localization of the AIR protein in a few cells in lymph node medulla.

Figure 8 shows the phenotypic characterization of the APECED reactive cells in thymus by double-immunofluorescence. The AIR protein is seen as red colour in the nuclei, forming typical speckled pattern with nuclear dots. In Figure 8A, the co-staining is with an antibody recognizing low molecular weight markers (AE1). The APECED positive cells fall into two types, one is expressing cytokeratin and is thus epithelial cell, the other one is non-epithelial and do not co-express cytokeratins. In Figure 8B an APECED

positive cell co-epresses a marker (CD83) typical for cells belonging to monocyte-macrophage-dendritic cell lineage.

Figure 9 shows the expression of the AIR protein, demonstrated by immunofluorescence, in mature, activated dendritic cells from peripheral blood. The expression of the AIR protein shows as distinct dots in the nuclei of dendritic cells.

Detailed description of the invention

The present invention is based on studies aiming for the identification and characterization of the gene defect in APECED. In the sequence studies, a cosmid/BAC (bacterial artificial chromosome) contig of 520 kb covering four gene markers D21S1460-D21S1912-PFKL-D21S154 [Kudoh, J. *et al.*, DNA Res. 4 (1997) 45-52] was constructed, and genomic sequencing in this region was performed [Kawasaki, K. *et al.*, Genome Res. 7 (1997) 250-261]. From this genomic sequence information the distance between D21S1912 and PFKL was determined to be approximately 140 kb (Fig. 1).

Using a computer program, such as GRAIL and GENSCAN [Uberbacher, E. C. and Mural, R. J., Proc. Natl. Acad. Sci. USA 88 (1991) 11261-11265; Burge, C. and Karlin, S., J. Mol. Biol. 268 (1997) 78-94], gene screening in the partial sequencing data within this region was performed. GENSCAN predicted several genes between D21S1912 and PFKL. One of these genes located just proximal to the PFKL gene contained the previously trapped exon HC21EXc33 [Kudoh, J. *et al.*, DNA Res. 4 (1997) 45-52] or MDC04M06 [Chen, H. *et al.*, Genome Res. 6 (1996) 747-760]. A set of primers for polymerase chain reaction (PCR) was then designed from the predicted exons. The PCR screening of various cDNA libraries using these primers allowed the isolation of a cDNA clone containing the exon HC21EXc33 (exon 13) from the thymus cDNA library (Fig. 2A).

A 3'-rapid amplification of cDNA ends (3'-RACE) and 5'-RACE using Marathon^{TM} cDNA Amplification Kit (Clontech Laboratories Inc, California, USA) according to manufacturer's protocol from the thymus cDNA library was performed using a primer c33F (sequence id. no. 7) and a primer 1R (sequence id. no. 8), respectively.

Sequencing analysis revealed a unique sequence of 2027 bp in overlapping PCR products that contains a 1635-bp open reading frame (ORF) from methionine at nt 128 to a TAG stop codon at nt 1763 encoding a predic-

ted novel protein designated AIR-1, for autoimmune regulator 1. AIR-1 encodes a protein of 545 amino acids with a predicted isoelectric point of 7.32 and a calculated molecular mass of 57,723 (Fig. 2B).

A 5'-RACE from the thymus cDNA using a primer 4R (sequence id. no. 9) resulted in an alternatively spliced product. Furthermore, two types of the cDNA clones were amplified with a primer pair 3F/c33R (sequence id. no. 10/sequence id. no. 11) and these clones encode for AIR-2 and AIR-3 proteins, sequence id. no. 4 and sequence id. no. 6, respectively (Fig. 2A) (sequence id. no. 3 and sequence id. no. 5). The AIR-2 and AIR-3 proteins consist of 348 and 254 amino acids, respectively (Fig. 2B). These results suggest that the APECED gene is transcribed as at least three types of mRNA by alternative splicing and/or use of an alternative 5' exon within the gene. RT-PCR analysis [Griffin, H. G. and Griffin, A. M., PCR Technology. Current Innovations, CRC Press, 1994] revealed that the AIR-1 transcript is also expressed in fetal liver (data not shown).

The APECED gene is approximately 13-kb in length and contains 15 exons, including the exon 1' specific to AIR-2 and AIR-3. It is transcribed in the direction of centromere to telomere (Figs 1, 2A). Based on this information, PCR primers were designed to amplify each exon from the genomic DNA and a mutation analysis of Swiss and Finnish APECED families was performed. Sequence comparison identified two mutations in the APECED gene of the patients (Fig. 3). The first mutation changes an Arg codon (CGA) to a stop codon (TGA) at amino acid position 257 in exon 6. This mutation was designated as R257stop mutation. The second mutation is a missense mutation that derived from the maternal chromosome in one Finnish patient (NP): a Lys codon (AAG) changes to a Glu codon (GAG) at amino acid position 42 in exon 2. This mutation is designated as K42E mutation (Figs 2A, 3C).

The R257stop mutation destroys a *Taq*I restriction enzyme site and the K42E mutation introduces a novel *Taq*I site. Thus these two mutations can be easily demonstrated in one or both alleles by *Taq*I digestion or by digestion using another enzyme cleaving at the recognition site 5'-TCGA-3'(Fig. 4).

The AIR-1 protein has strong homology in certain domains to the major autoantigens (Mi-2) associated with the autoimmune disease dermatomyositis [Seeig, H. P. *et al.*, Arthritis Rheum. 38 (1995) 1389-1399; Ge, Q. *et al.*, J. Clin. Invest. 96 (1995) 1730-1737], Sp140, a protein from the nuclear body, an organelle involved in the pathogenesis of certain types of

leukemia, and which is also the target of antibodies in the serum of patients with the autoimmune disease primary bilary cirrhosis [Bloch, D. B. *et al.*, J. Biol. Chem. 271 (1996) 29198-29204]. In addition, the homologies extend to other nuclear proteins such as TIF1 [Le Douarin, B. *et al.*, EMBO J. 14 (1995) 2020-2033], LYSP100 [Dent, A. L. *et al.*, Blood 88 (1996) 1423-1426], and putative yeast and *C. elegans* proteins. The AIR-1 protein homologies are principally in two PHD finger motifs (amino acid 299 to 340 and 434 to 475) (Fig. 5). AIR-1 also contains a proline-rich region (amino acid 350 to 430) (Fig. 2B). The PHD finger is a cysteine-rich structure that is distinguished from the RING finger (C3HC4) and LIM domain (C2HC5) because it contains a consensus of C4HC3. [Aasland, R. *et al.*, Trends Biochem. Sci. 20 (1995) 56-59]. The PHD finger motif is found in a number of chromatin-associated proteins such as HRX that is involved in the t(11:17) translocation in acute leukemia [Chaplin, T. *et al.*, Blood 86 (1995) 2073-2076]. The proline-rich region is assumed to be involved in protein-protein interaction or DNA binding. The presence of the PHD finger and proline-rich regions indicates a function for AIRs as transcription regulatory proteins. However, the AIR proteins have no apparent nuclear translocation signal, and thus other proteins containing such signal may interact with AIR to translocate it to the nucleus. In fact, the AIR proteins also have the LXXLL motif that is a signature sequence to bind to nuclear receptors [Heery, D. M. *et al.*, Nature 387 (1997) 733-736] (Fig. 2B).

The clinical picture of APECED and the observed immunological abnormality with strong autoimmune response towards several target organs and antigens suggest that the product of the APECED gene has a central role in immune (ontogeny) maturation and regulation of immune response towards self and nonself.

According to the diagnostic method of the invention, the presence of the defective APECED gene can be detected from a biological sample by any known detection method suitable for detecting mutations. Such methods include the method described by Saiki *et al.* [Proc. Natl. Acad. Sci USA 86 (1989) 6230-6234) utilizing hybridization to an allele specific oligonucleotide probe, or modifications thereof; the method described by Newton, C. R. *et al.* [Nucl. Acids Res. 17 (1989) 2503-2516] using the DNA sequences or DNA-fragments of the invention as probes; the solid phase minisequencing method described by Syvänen *et al.* [Genomics 8 (1990) 684-692] in which use is made of a biotinylated probe; or the oligonucleotide ligation method described

by Landegren, U. *et al.* [Science 241 (1988) 1077-1080]. Methods include the denaturing gradient gel electrophoresis (DGGE) [Fischer, S.G. and Lerman, L.S., PNAS 80 (1983) 1579-1583] or a modification of this method, constant denaturant gel electrophoresis (CDGE) [Hoving *et al.*, Genes Chromosomes Cancer 5 (1992) 97-103]. The mutation separation principle of DGGE and CDGE is based on the melting behavior of the DNA double helix of a given fragment.

Since the mutations of the APECED gene involve a site sensitive to *Taq*I digestion, the mutation are preferably detected in one or both alleles by *Taq*I digestion or by digestion using another enzyme cleaving at recognition site 5'-TCGA-3' The chemical mismatch cleavage for mutation analysis can be used [Grompe, M. *et al.*, Proc. Natl. Acad. Sci. USA 86(15)(1989) 5888-5892].

In the diagnostic method of the invention the biological sample can be any tissue or body fluid containing cells, such as blood, e.g. umbilical cord blood, separated blood cells, such as lymphocytes, B-cells, T-cells etc., biopsy material, such as fetal liver or thymus biopsy, sperm, saliva, etc. The biological sample can be, where necessary, pretreated in a suitable manner known to those skilled in the art.

When the DNA sequence of the present invention is used therapeutically any techniques presently available for gene therapy can be employed. Accordingly, in the technique known as *ex vivo* therapy patient cells (e.g. umbilical cord blood from the fetus) with the defective gene are taken from the patient, DNA sequences encoding the normal (healthy) gene product incorporated in a carrier vector are transducted or transfected to the cells and the cells are returned to the patient. If the techniques known as *in situ* therapy is used, the DNA sequences encoding the normal gene product are first inserted to a suitable carrier vector, and the carrier is then introduced to the affected tissue, such as peripheral blood, liver or bone marrow. The carrier vector used can be a retrovirus vector, an adeno virus vector, an adeno associated virus (AAV) vector or an eucaryotic vector. The therapy can be performed intra utero or during adult life. Depending on the cells to be treated these techniques lead either to a transient cure, where cells from affected organ are treated, or to a permanent cure, in case of the treatment of stem cells.

The present invention provides means for an easy and more rapid diagnosis of the diseases related to immune maturation and regulation of

immune response towards self and nonself, such as APECED, and, specifically, enables prenatal diagnosis and carrier diagnosis. Furthermore, it provides a background for therapy.

The invention is now elucidated by the following non-limiting examples.

Example 1

Localization of the APECED gene

Genomic sequencing of cosmid DNAs was performed by the shotgun method described by Kawasaki, K. *et al.*, Genome Res. 7 (1997) 250-261. Cosmids D1G8, D40G11, D9G11, D28B11, and D4G11 and gene marker D21S1912 are described by Kudoh, J. *et al.*, DNA Res. 4 (1997) 45-52].

cDNA cloning

The phage DNAs prepared from human thymus cDNA library (Clontech, HL1127a) were used as a PCR template. 20 ng of phage DNA which represents approximately $4x10^6$ phages was added to a 10 ml of reaction mixture containing 1x buffer [16mM $(NH_4)_2SO_4$, 50mM Tris-HCl, pH 9.2, 1.75 mM $MgCl_2$, 0.001% (w/v) gelatin), 0.2 mM each of dNTPs, 1M Betaine (Sigma), 0.35 U of Tap and Pwo DNA polymerase (EXpand Long Template PCR System, Boehringer Mannheim), and 0.5 mM of each of the primers, 2F and c33R, 2F and 4R, and 2F' and 2R', respectively.

The cDNA fragment was amplified by PCR using the following conditions: 94°C for 3 min., 35 cycles of 94°C for 30 sec, 60 °C for 30 sec in 2F/c33R and 2F/4R or 65°C for 30 sec in 2F'/2R', and 68°C for 90 sec. 3'- and 5'-RACE were carried out by Marathon cDNA Amplification Kit (Human Thymus; Clontech). PCR reaction was performed in a 10 µl volume containing 1x buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin), 0.2 mM each of dNTPs, 0.25 U of AmpliTaq Gold polymerase (Perkin-Elmer), and 0.5 mM of each of the exon-specific primers. 3'-RACE product was amplified by PCR with the following conditions: 95°C for 9 min., 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec.

The cDNA fragments were sequenced by the dye deoxy terminator cycle sequencing method (according to ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit protocol P/N 402078, Perkin Elmer Corporation, California) using specific primers, 2F and c33R, and AmpliTaq/FS

DNA polymerase (Perkin-Elmer), and then analyzed by using an automatic DNA sequencer (Applied Biosystems 377). Primer sequences used were

1R: 5'-GTTCCCGAGTGGAAGGCGCTGC-3' (sequence id. no. 8)

2F: 5'-GGATTCAGACCATGTCAGCTTCA-3' (sequence id. no. 12)

3F: 5'-GAGTTCAGGTACCCAGAGATGCTG-3' (sequence id. no. 10)

c33R: 5'-CTCGCTCAGAAGGGACTCCA-3' (sequence id. no. 11)

4R: 5'-AGGGGACAGGCAGGCCAGGT-3' (sequence id. no. 9)

2F': 5'-GTGCTGTTCAAGGACTACAAC-3' (sequence id. no. 13)

2R': 5'-TGGATGAGGATCCCCTCCACG-3' (sequence id. no. 14)

AP1: 5'-CCATCCTAATACGACTCACTATAGGGC-3' (sequence id. no. 15) and

c33F: 5'-GATGACACTGCCAGTCACGA-3' (sequence id. no. 7).

Example 2

Mutation analysis of the APECED gene

For the mutation analysis the DNA samples were purified from periferal blood mononuclear cells from patients with APECED and from suspected carriers of APECED and from normal healthy controls (according to Sambrook *et al.* 1989, Molecular Cloning. A Laboratory Manual. CSH Press) and subjected to PCR using primers specific for all identified exons.

For sequencing the mutated exons, PCR fragments, 6F/6R in exon 6 and 49300F/49622R in exon 2, were amplified by PCR with the following conditions: 95°C for 9 min., 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, and 94°C for 3 min., 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 68°C for 30 sec, respectively. The PCR products were sequenced using specific primers

6F: 5'-TGCAGGCTGTGGGAACTCCA-3' (sequence id. no. 16)

6R: 5'-AGAAAAAGAGCTGTACCCTGTG-3' (sequence id. no. 17)

3R: 5'-TGCAAGGAAGAGGGGCGTCAGC-3' (sequence id. no. 18)

49300F: 5'-TCCACCACAAGCCGAGGAGAT-3' (sequence id. no. 19) and 49622R: 5'-ACGGGCTCCTCAAACACCACT-3' (sequence id. no. 20).

In the mutation analysis by sequencing, two Swiss and three Finnish (HP1, HP2 and MP) patients with APECED were homozygous for R257stop allele, whereas one Finnish patient (NP) was heterozygous for this mutation (Fig. 3A, B). The R257stop mutation of NP was derived from the

paternal chromosome. The second mutation, K42E mutation, was found in one Finnish patient (NP): a Lys codon (AAG) changes to a Glu codon (GAG) at amino acid position 42 in exon 2.(Figs 2A, 3C). This mutation derived from the maternal chromosome.

Example 3

Restriction enzyme TaqI analysis of two mutations in exons 2 and 6 of APECED gene

Analysis of the mutation sites in exons 2 and 6 in large series of individuals was performed using the restriction enzyme TaqI.The TaqI digestion for exons 2 and 6 was done as follows. Ten microlitres of amplification product was incubated at 65°C for 1 hour in 20 µl of reaction mixture containing 1x TaqI digestion buffer (New England Biolabs, NY, 100 µl/ml of BSA and 10U of TaqI enzyme (New England Biolabs, NY). After the digestion fragments were separated in 1.5% agarose gel and visualized by EtBr staining.

For exon 2, the fragment containing the mutation site K42E was amplified with primers GR1/2F and GR1/2R with the following conditions: 95°C for 3 min., 35 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min. The 1x reaction mix used contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin), 0.2 mM each of dNTPs, 0.25 U of Dynazyme (Finnzymes, Finland), and 0.5 mM of each of the exon-specific primers. The normal allele produces a 312 bp fragment whereas the mutated allele gives a 133 bp and a 179 bp fragment. Primer sequences for GR1/2F and GR1/2R are 5'-TGGAGATGGGCAGGCCGCAGGGTG (sequence id. no. 21) and 5'-CAGTCCAGCTGGGCTGAGCAGGTG (sequence id. no. 22), respectively.

For exon 6, the fragment containing the R257stop mutation site was amplified with primers GR1/5IF and GR1/5IR with the same conditions described for exon 2 (see above). The normal allele produces a 225 bp fragment whereas the mutated allele gives a 285 bp fragment. Primer sequences for GR1/5IF and GR1/5IR are 5'-GCGGCTCCAAGAAGTGCATCCAGG (sequence id. no. 23) and 5'-CTCCACCCTGCAAGGAAGAGGGGC (sequence id. no. 24), respectively.

The screening of 50 Finnish and 50 Swiss healthy individuals did not reveal R257stop or K42E mutations by *Taq*I digestion. Similarly, PCR ana-

lysis of 20 unaffected Japanese was performed and no mutations were found in any of these positions. These results demonstrate that the APECED gene is responsible for the pathogenesis of APECED.

Mutations were found in the AIR-1 transcript but not in the AIR-2 and AIR-3 transcripts from all the APECED patients tested. Two Swiss and three Finnish (HP1, HP2 and MP) patients who are homozygous for the R257stop mutation completely lack functional AIR-1 protein but still have intact AIR-2 and AIR-3 proteins.

One common mutation seems responsible for the genetic defect in approximately 90% of the Finnish APECED cases and a haplotype analysis with the markers D21S141, D21S1912 and PFKL shows that the R257stop mutation is likely to be this common mutation [Björses, P. *et al.*, Am. J. Hum. Genet. 59 (1996) 879-886].

Example 4

Analysis of the AIR protein expression

In this example, synthetic peptides representing amino-acid sequences of the AIR-1 protein, were used to generate a polyvalent mouse antiserum against the AIR-1 protein.

For the peptide synthesis, two peptides were chosen according to the antigenicity prediction by Pepsort program (GCC package, Wisconsin, USA). The peptides AIR-1/2 and AIR-1/6 (TLHLKEKEGCPQAFH, sequence id. no. 25 and GKNKARSSSGPKPLV, sequence id. no. 26, respectively) representing exons 2 and 6, respectively, of the APECED gene were synthesized onto a branched lysine core (Fmoc8-Lys4-Lys2-Lys-betaAla-Wang resin, Calbiochem-Novabiochem, La Jolla, Ca, USA) resulting in an octameric multible antigen peptide (MAP) [Tam, J. P. *et al.*, Proc. Natl. Acad. Sci. USA 85 (1988) 5409-5413; Adermann, K. *et al.*, in Solid Phase Synthesis, Biological and Biomedical Applications, pp. 429-432, Ed. R. Epton, Mayflower Worldwide Ltd., Birmingham, 1994], Syntheses were performed by Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a simultaneous multiple peptide synthesizer (SMPS 350, Zinsser Analytic, Frankfurt, Germany). Purity of MAPs was analyzed by reverse-phase HPLC (System Gold, Beckman Instruments Inc, Fullerton, CA, USA).

To obtain murine polyclonal antibodies, eight-week old Balb/c mice were immunized with an intraperitoneal injection of 25 micrograms of each

peptide in 0,4 ml of a 1:1 mixture of Freund's Complete Adjuvant (Difco Laboratories, Detroit, MI, USA) and physiological saline (NaCl, 0,15 M). One month later the animals were boosted with an intramuscular injection of 35 micrograms of antigens in Freund's incomplete adjuvant and saline (1:1) (0,2 ml were distributed into four sites). Three weeks later the peptides in a dose of 50 micrograms/mouse were administered intravenously and sera were obtained 7 days later.

For the production of EBV transformed B-cells, peripheral blood leukocytes were obtained from healthy control persons. The B-cells were transformed with EBV (Epstein-Barr virus) using standard protocol, and the cell lines were maintained in RPMI 1640, supplemented with 10% FCS (fetal calf serum). An aliquot of cells were stimulated for 12 hours with 10 mg/ml of phytohemagglutinin (PHA) to obtain mitogen-activated T-cells.

Tissue samples were obtained from stillborn fetuses at six months gestational age. Fetal liver, spleen, thymus and lymphnodes were homogenized, the homogenates were cleared with centrifugations (20 000 rpm for 20 minutes) and the samples were used for western blot analysis.

For analysis of polyclonal sera, Elisa and western blot analysis were performed. Microtitre ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with the peptides (1 micrograms /well in PBS, pH 7,5) at $4^{o}C$ overnight and blocked with 2 % of BSA in PBS. The plates were then incubated with titrated mouse immune sera and normal (control) sera at room temperature for 4 h. Finally the bound peptide-specific antibodies were detected by use of anti-mouse HRP-labelled immunoglobulins (Dako A/S, Denmark) essentially as previously described [Ovod, V. A. *et al.*, AIDS 6 (1992) 25-34].

For western blotting, tissue homogenates, EBV transformed B-cells or PHA-activated T-cells were boiled for 10 minutes in 2x sample buffer (for tissue homogenates: 100 microliters of homogenate mixed with 100 microliters of sample buffer; for cells: one million cells/100 ml of buffer) and analyzed in western blotting as described in Ovod, V. A. *et al., supra.*

The antisera so produced reacted with the AIR-1-protein low amount in normal fetal spleen, thymus and lymphonode as well as, in EBV-transformed B-cells and in PHA-activated T-cells. In the ELISA assay towards the immunogenic peptides, all four mice gave a strong reactivity towards the peptide used for the immunization. In the western blotting analysis using either

the tissue homogenates or stimulated T-cells or established B-cells, a strong band of approx. 60 kD molecular weight was seen in fetal liver (Fig. 6), while weaker bands of the same size were seen in the other samples.

Example 5

Identification of the expression of APECED in thymus and other lymphoid organs

mRNA *in situ* hybridization and immunohistochemistry were used to identify APECED-expressing cells in various normal fetal and adult human tissues. Thymus samples were obtained in conjunction of corrective surgery from cardiac patients aged 2-19 years. Other tissue samples were obtained from surgical biopsy or from autopsy material. This was approved by Hospital Ethics Committees at Tampere University Hospital and Helsinki University Central Hospital. The tissue materials were stored frozen or formaldehyde fixed and paraffin embedded until used.

For mRNA *in situ* hybridization, three cDNA fragments for riboprobes were amplified by RT-PCR from thymus mRNA (Clontech) with primer pairs: 5'-ATG GCG ACG GAC GCG GCG CTA CGC-'3 (seq. id. no. 27) and 5'-CCT GGA TGT ACT TCT TGG AGC CGC-3' (seq. id. no. 28), 5'-GAG CCC GAG GGG CCG TGG AGG GGA-3' (seq. id. no. 29) and 5'-GGC TGC ACC TCC TGG ACT GTT GCC-3' (seq. id. no. 30), and 5'-GAT CCT GCT CAG GAG ACG TGA CCC-3' (seq. id. no. 31) and 5'-CAC CAG GCA AGG AGA GGC TCC CGG-3' (seq. id. no. 32), designed to amplify fragments spanning nucleotides 137 - 812, 738 - 1185 and 1554 - 2009 of the sequence id. no. 1, respectively. The amplified fragments were subcloned into a pCRII-TOPO vector (Invitrogen).

For in vitro transcription the plasmids were linearized and sense and antisense probes were synthesized with digoxigenin-UTP as described (Boehringer Mannheim Nonradioactive *in situ* Hybridization Application Manual). Labeled probes were purified with MicroSpinG-50 columns (Pharmacia Biotech). The pretreatment and hybridization of formaldehyde fixed, paraffin embedded tissue sections were performed as described by H. Breitschopf and G. Sucharek. (Boehringer Mannheim Nonradioactive *in situ* Hybridization Application Manual, Detection of mRNA on paraffin embedded material of the central nervous system with DIG-labeled RNA probes, pp 136-138.)

For the preparation of antibodies to the AIR protein, the APECED cDNA (sequences 137 - 1774 of sequence id. no. 1) containing a full-coding region was amplified from Marathon human thymus cDNA (Clontech) with primers ExF and ExR2. The primer sequences for ExF and ExR2 were 5'-CCA CCC CAT GGC GAC GGA CG-3' (sequence id. no. 33) and 5'-GGA ATT CGG AGG GGA AGG GGG CCG CCG GA-3' (sequence id. no. 34). The amplified cDNA was digested with NcoI and EcoRI and cloned (pHPAIRE) into pET32a vector (Novagen). The protein was expressed in *E. coli* and purified by His-tag as described by manufacturer (QiaExpress Type IV Kit, Cat No 32149, Qiagen, USA).

To obtain murine polyclonal antibodies, Balb/c mice were immunised essentially as described in Example 4 using 100 micrograms of the bacterially expressed AIR protein with booster doses of 25 and 25 micrograms.

Japanese white rabbits were immunised with a synthetic peptide representing amino acids 526-545 (DGILQWAIQSMARPAAPFPS, sequence id. no. 36) of sequence id. no. 2. The specificities of the antisera were checked with ELISA and Western blotting using standard procedures.

For immunocytochemistry, frozen sections of tissue samples were fixed for 20 min in 4% paraformaldehyde. The AIR antibody (rabbit or mouse) in an appropriate dilution was incubated for 30 min at 37°C, with a biotin conjugated anti-mouse or anti–rabbit secondary antidody (Vector, CA, USA). The biotinylated antibody was revealed by incubating with Texas Red-avidin (Vector, CA, USA) for 30 min at 37°C.

With in situ hybridization, a positive signal was seen in a few cells in thymus medulla (Fig. 7A). The APECED *in situ* -positive cells were infrequent and scattered as single cells in the medulla, but occasionally one or two APECED-expressing cells were seen adjacent to or buried into the Hassal's corpuscles that represent conglomerates of medullary epithelial cells. In the positive cells, APECED mRNA was predominantly localized in the cell nucleus. In human adult lymph node tissues, infrequent cells expressed APECED mRNA in the medulla and occasionally in the paracortical region, too (Fig. 7B) No hybridization signal was seen in the germinal centers.

Immunohistochemistry with mouse and rabbit polyclonal antisera to the AIR protein showed strong reactivity with selected cells in thymus medulla, lymph nodes and fetal liver (Fig. 7C and 7D) The comparison of the reaction

pattern obtained by immunohistochemistry to that obtained by in situ hybridization clearly established that specific, rare cells in thymus medulla and lymph node medulla and paracortex express APECED mRNA and the AIR protein. By either method, neither mRNA nor protein was detected in other adult tissues studied, including the target organs for tissue destruction in APECED (adrenal glands, parathyroid glands, gonads). In human fetal tissues, APECED positive cells were seen, although extremely infrequently, in the stroma of placental chorionic villi and in the sinusoidal area of the liver. In the fetal liver, the APECED positive cells were often localized pairwise like mirror images, suggesting that the cells were undergoing mitosis. Rare APECED expressing cells were also found in fetal thymus but the expression was not observed in other fetal tissues.

At the subcellular level, the AIR protein localized in small nuclear dots in the adult thymus, giving a characteristic speckled pattern (Fig. 7C; and Fig. 8A and 8B), but localized in the cytoplasm of cells in lymph nodes. In the rare positive cells in fetal liver, many of which were mitotic, the AIR protein was localized in the cytoplasm.

Example 6

Characterization of the phenotype of the APECED positive cells in thymus

Double staining with two antibodies was used to further characterize the cell type expressing APECED gene. In view of the fact that dendritic cells (DC) and thymus epithelium are both involved in the regulation of immune maturation, expression of markers for these cells were studied.

For double immunofluorencence detection the AIR staining was performed as described in example 5 with rabbit anti-AIR serum. The slides were then incubated with a second primary antibody [AE1 (Neomarkers, CA, USA), AE3 (Neomarkers, CA, USA), CD11c (Immunotech, France), or CD83 (Immunotech, France)] in an appropriate dilution for 30 min at 37°C, and the reaction was revealed by incubating with a FITC conjugated secondary anti-mouse antibody (Vector, CA, USA) for 30 min at 37°C.

Antibodies reacting with low molecular weight basic (AE1) or high molecular weight acidic (AE3) cytokeratins stained the thymus in a reticular fashion, and the APECED positive cells were seen either buried into this net or in close apposition with the keratin-positive cells. Confocal microscopy

clearly demonstrated that some of the APECED positive cells were cytokeratin positive while some remained negative (Figure 8A). A co-localization was stronger with AE1 than with AE3. The distribution of epithelial (AE1 positive) and non-epithelial APECED expressing cells varied but in most thymus preparates more than half were epithelial.

Less than half of the APECED expressing cells in thymus stained with markers CD11c and CD83 that react with cells of the monocyte-macrophage-dendritic cell lineage. In most cases, the staining reaction was weak but a few cells showed an intensive staining with the given marker (Fig. 8B). CD83 costained 5 to 40 % of the APECED positive cells. Antibody CD11c, reported to be specific for mature dendritic cells, reacted with up to 5 - 10 % of the APECED positive cells. All APECED positive cells were strongly positive for HLA-DR staining, however (data not sown).

These results suggest that in thymus the APECED gene is in fact expressed in two distinct cell populations, one epithelial and the other non-epithelial. The latter cell type is likely the one also expressing the APECED gene in extrathymic lymphoid tissues.

Example 7

APECED expression in stimulated dendritic cells *in vitro*

To show an APECED expression in dendritic cells derived from peripheral blood monocytes that are DC precursors, these cells were cultured at the presence of cytokines using conditions that are known to lead to the expansion and maturation of dendritic cells.

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque centrifugation, and adherent cells were separated and cultured in the presence of human recombinant GM-CSF (1000 units/ml) and rhIL-4 (1000 units/ml, both from R&D Systems), as described [Schuler, G. and Romani, N., Adv. Exp. Med. Biol. 417 (1997) 7 - 13]. Cells were further cultured for three days with 1/4 V/V of macrophage conditioned media. Cells were harvested at two days intervals and samples were prepared for RT-PCR. For RT-PCR total RNA was purified from DCs by using a commercial kit from Clontech (USA) (Nucleospin RNA Kit) according to manufacturer's instructions. An aliquot of RNA was transferred into cDNA with a commercial kit from Pharmacia (Sweden) (First-strand Synthesis Kit) and PCR for this DNA sample was performed. For PCR the fragment was amplified with primers 5'- GAT CCT

GCT CAG GAG ACG TGA CCC-3' (seq. id. no. 31; 1554 -1577 of seq. id. no. 1) and 5'-GGA CTG AGG AAG GAG GTG TCC TTC -3' (seq. id. no. 35; 1818-1841 of seq. id. no. 1) with the following conditions: 35 cycles of 95°C for 1 min., 62°C for 30 sec and 72°C for 1 min. The 1x reaction mix contained 50mM KCl, 10mM Tris-HCl, pH8.3, 1.5mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2mM each of dNTPs, 0.25 U of Dynazyme (Finnzymes, Finland). A fragment of 287bp was detected by 1.5% agarose electrophoresis.

Cytospin preparations were further made for immunohistochemistry.

During this 7 to 10 days culture period approximately half of the cells developed the characteristic veiled morphology of DC and their phenotypic cell markers (CD11cand CD83) corresponded to mature DCs (Figure 9). The APECED expression was studied by RT-PCR and immunocytochemistry at two to three days intervals. In the starting material, i.e. the adherent cell pool from peripheral blood, no APECED expression was found. After seven days of culture in the presence of GM-CSF and IL-4, RT-PCR showed APECED mRNA expression and immunofluorescence showed a few AIR specific nuclear dots. After an additional 3-day-culture with conditioned medium from macrophage cultures a strong speckled pattern of nuclear AIR expression was seen (Figure 9A). The RT-PCR analysis of the mature (10 days) culture confirmed the AIR protein expression.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Kai Krohn et al.
(B) STREET: Iltarusko, Salmentaantie 751
(C) CITY: 36450 Salmentaka
(E) COUNTRY: Finland
(F) POSTAL CODE (ZIP): none

(ii) TITLE OF INVENTION: Novel Gene

(iii) NUMBER OF SEQUENCES: 26

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2036 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:137..1774
(D) OTHER INFORMATION:/product= "AIR-1"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:137..1771
(D) OTHER INFORMATION:/product= "AIR-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Gln Glu Thr Leu His Leu Lys Glu Lys Glu Gly Cys Pro Gln Ala Phe
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                80                  85                  90

CGG CTG CAG CCC ATC CTG GAC AGC TTC CCC AAA GAT GTG GAC CTC AGC      457
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            95                  100                 105

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Gln Pro Arg Lys Gly Arg Lys Pro Pro Ala Val Pro Lys Ala Leu Val
        110                 115                 120

CCG CCA CCC AGA CTC CCC ACC AAG AGG AAG GCC TCA GAA GAG GCT CGA      553
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    125                 130                 135

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140                 145                 150                 155

TCT CAA CTG AAG GCC AAG CCC CCC AAG AAG CCG GAG AGC AGC GCA GAG      649
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                160                 165                 170

CAG CAG CGC CTT CCA CTC GGG AAC GGG ATT CAG ACC ATG TCA GCT TCA      697
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            175                 180                 185

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Val Gln Arg Ala Val Ala Met Ser Ser Gly Asp Val Pro Gly Ala Arg
        190                 195                 200

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Gly Ala Val Glu Gly Ile Leu Ile Gln Gln Val Phe Glu Ser Gly Gly
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300                 305                 310                 315

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            335                 340                 345

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Val Gln Pro Arg Ala Glu Glu Pro Arg Pro Gln Glu Pro Pro Val Glu
        350                 355                 360

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    365                 370                 375

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Ser Gly Asp Val Thr Pro Ala Pro Val Glu Gly Val Leu Ala Pro Ser
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            495                 500                 505

GAG CCC GCT CTG CAC AGG GAT GAC CTG GAG TCC CTT CTG AGC GAG CAC    1705
Glu Pro Ala Leu His Arg Asp Asp Leu Glu Ser Leu Leu Ser Glu His
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ACC TTC GAT GGC ATC CTG CAG TGG GCC ATC CAG AGC ATG GCC CGT CCG    1753
Thr Phe Asp Gly Ile Leu Gln Trp Ala Ile Gln Ser Met Ala Arg Pro
    525                 530                 535

GCG GCC CCC TTC CCC TCC TGA CCCCAGATGG CCGGGACATG CAGCTCTGAT       1804
Ala Ala Pro Phe Pro Ser  *
540                 545

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 545 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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        35                  40                  45
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Leu Asp Ser Phe Pro Lys Asp Val Asp Leu Ser Gln Pro Arg Lys Gly
100 105 110

Arg Lys Pro Pro Ala Val Pro Lys Ala Leu Val Pro Pro Pro Arg Leu
115 120 125

Pro Thr Lys Arg Lys Ala Ser Glu Glu Ala Arg Ala Ala Ala Pro Ala
130 135 140

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180 185 190

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275 280 285

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385                 390                 395                 400

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                405                 410                 415

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            420                 425                 430

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        435                 440                 445

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                485                 490                 495

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Ser *
545
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1545 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:237..1283
(D) OTHER INFORMATION:/product= "AIR-2"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:237..1280
(D) OTHER INFORMATION:/product= "AIR-2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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 1               5                  10                  15

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            20                  25                  30

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        35                  40                  45

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Pro Gly Ser Met Gly Ala Gly Gln Arg Leu Gly Ser Ser Gly Thr Gln
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65                  70                  75                  80

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                85                  90                  95

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Lys Asn Glu Asp Glu Cys Ala Val Cys Arg Asp Gly Gly Glu Leu Ile
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225                 230                 235                 240

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    290                 295                 300

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AACAGCTCTG TGTTTCTGGG GACACCAGCC ATCATGTGCC TGGAAATTAA ACCCTGCCCC 1473

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 348 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Trp Leu Val Tyr Ser Ser Gly Ala Pro Gly Thr Gln Gln Pro Ala
1 5 10 15

Arg Asn Arg Val Phe Phe Pro Ile Gly Met Ala Pro Gly Gly Val Cys
20 25 30

Trp Arg Pro Asp Gly Trp Gly Thr Gly Gly Gln Gly Arg Ile Ser Gly
35 40 45

Pro Gly Ser Met Gly Ala Gly Gln Arg Leu Gly Ser Ser Gly Thr Gln
50 55 60

Arg Cys Cys Trp Gly Ser Cys Phe Gly Lys Glu Val Ala Leu Arg Arg
65 70 75 80

Val Leu His Pro Ser Pro Val Cys Met Gly Val Ser Cys Leu Cys Gln
85 90 95

Lys Asn Glu Asp Glu Cys Ala Val Cys Arg Asp Gly Gly Glu Leu Ile
100 105 110

Cys Cys Asp Gly Cys Pro Arg Ala Phe His Leu Ala Cys Leu Ser Pro
115 120 125

Pro Leu Arg Glu Ile Pro Ser Gly Thr Trp Arg Cys Ser Ser Cys Leu
130 135 140

Gln Ala Thr Val Gln Glu Val Gln Pro Arg Ala Glu Glu Pro Arg Pro
145 150 155 160

```
Gln Glu Pro Pro Val Glu Thr Pro Leu Pro Pro Gly Leu Arg Ser Ala
                165                 170                 175

Gly Glu Glu Val Arg Gly Pro Pro Gly Glu Pro Leu Ala Gly Met Asp
            180                 185                 190

Thr Thr Leu Val Tyr Lys His Leu Pro Ala Pro Pro Ser Ala Ala Pro
        195                 200                 205

Leu Pro Gly Leu Asp Ser Ser Ala Leu His Pro Leu Leu Cys Val Gly
    210                 215                 220

Pro Glu Gly Gln Gln Asn Leu Ala Pro Gly Ala Arg Cys Gly Val Cys
225                 230                 235                 240

Gly Asp Gly Thr Asp Val Leu Arg Cys Thr His Cys Ala Ala Ala Phe
                245                 250                 255

His Trp Arg Cys His Phe Pro Ala Gly Thr Ser Arg Pro Gly Thr Gly
            260                 265                 270

Leu Arg Cys Arg Ser Cys Ser Gly Asp Val Thr Pro Ala Pro Val Glu
        275                 280                 285

Gly Val Leu Ala Pro Ser Pro Ala Arg Leu Ala Pro Gly Pro Ala Lys
    290                 295                 300

Asp Asp Thr Ala Ser His Glu Pro Ala Leu His Arg Asp Asp Leu Glu
305                 310                 315                 320

Ser Leu Leu Ser Glu His Thr Phe Asp Gly Ile Leu Gln Trp Ala Ile
                325                 330                 335

Gln Ser Met Ala Arg Pro Ala Ala Pro Phe Pro Ser  *
            340                 345
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1463 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:237..1001
(D) OTHER INFORMATION:/product= "AIR-3"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:237..998
(D) OTHER INFORMATION:/product= "AIR-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
AGAGAAAGTG AGGTCTTCTC AGGCTCTTAA GAGCATGGCG TTTGGTCCAG GCTGTACCCG    60

CTGCTCTCAG CTGGGCCCGT GGGTGGGCCG GGCGCCCCTG CTATAGCCAG GAGGTCAAGG   120

ATCCACTGGG AATGCCATGC TCATCTTTCG TCCCCAGCAT GGTTTCTTAA TGGGGTAGAA   180

GCAGGTCGGG AGAGACCTCC CTGGGCCTGG CCCCACTGCC CTGTGAGGAA GGGTTC       236

ATG TGG TTG GTG TAC AGT TCC GGG GCC CCT GGA ACG CAG CAG CCT GCA      284
Met Trp Leu Val Tyr Ser Ser Gly Ala Pro Gly Thr Gln Gln Pro Ala
 1               5                  10                  15

AGA AAC CGG GTT TTC TTC CCA ATA GGG ATG GCC CCG GGG GGT GTC TGT     332
Arg Asn Arg Val Phe Phe Pro Ile Gly Met Ala Pro Gly Gly Val Cys
            20                  25                  30

TGG AGA CCA GAT GGA TGG GGA ACA GGT GGT CAG GGC AGA ATT TCA GGC     380
Trp Arg Pro Asp Gly Trp Gly Thr Gly Gly Gln Gly Arg Ile Ser Gly
        35                  40                  45

CCT GGC AGC ATG GGA GCA GGG CAG AGA CTG GGG AGT TCA GGT ACC CAG     428
Pro Gly Ser Met Gly Ala Gly Gln Arg Leu Gly Ser Ser Gly Thr Gln
    50                  55                  60

AGA TGC TGC TGG GGG AGC TGT TTT GGG AAG GAG GTG GCT CTC AGG AGG     476
Arg Cys Cys Trp Gly Ser Cys Phe Gly Lys Glu Val Ala Leu Arg Arg
65                  70                  75                  80

GTG CTG CAC CCC AGC CCA GTC TGC ATG GGC GTC TCT TGC CTG TGC CAG     524
Val Leu His Pro Ser Pro Val Cys Met Gly Val Ser Cys Leu Cys Gln
                85                  90                  95

AAG AAT GAG GAC GAG TGT GCC GTG TGT CGG GAC GGC GGG GAG CTC ATC     572
Lys Asn Glu Asp Glu Cys Ala Val Cys Arg Asp Gly Gly Glu Leu Ile
            100                 105                 110

TGC TGT GAC GGC TGC CCT CGG GCC TTC CAC CTG GCC TGC CTG TCC CCT     620
Cys Cys Asp Gly Cys Pro Arg Ala Phe His Leu Ala Cys Leu Ser Pro
        115                 120                 125

CCG CTC CGG GAG ATC CCC AGT GGG ACC TGG AGG TGC TCC AGC TGC CTG     668
Pro Leu Arg Glu Ile Pro Ser Gly Thr Trp Arg Cys Ser Ser Cys Leu
    130                 135                 140

CAG GCA ACA GTC CAG GAG GTG CAG CCC CGG GCA GAG GAG CCC CGG CCC     716
Gln Ala Thr Val Gln Glu Val Gln Pro Arg Ala Glu Glu Pro Arg Pro
145                 150                 155                 160

CAG GAG CCA CCC GTG GAG ACC CCG CTC CCC CCG GGG CTT AGG TCG GCG     764
Gln Glu Pro Pro Val Glu Thr Pro Leu Pro Pro Gly Leu Arg Ser Ala
                165                 170                 175
```

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GGA GAG GAG CCC CGC TGC CAG GGC TGG ACT CCT CGG CCC TGC ACC CCC      812
Gly Glu Glu Pro Arg Cys Gln Gly Trp Thr Pro Arg Pro Cys Thr Pro
            180                 185                 190

TAC TGT GTG TGG GTC CTG AGG GTC AGC AGA ACC TGG CTC CTG GTG CGC      860
Tyr Cys Val Trp Val Leu Arg Val Ser Arg Thr Trp Leu Leu Val Arg
        195                 200                 205

GTT GCG GGG TGT GCG GAG ATG GTA CGG ACG TGC TGC GGT GTA CTC ACT      908
Val Ala Gly Cys Ala Glu Met Val Arg Thr Cys Cys Gly Val Leu Thr
    210                 215                 220

GCG CCG CTG CCT TCC ACT GGC GCT GCC ACT TCC CAG CCG GCA CCT CCC      956
Ala Pro Leu Pro Ser Thr Gly Ala Ala Thr Ser Gln Pro Ala Pro Pro
225                 230                 235                 240

GGC CCG GGA CGG GCC TGC GCT GCA GAT CCT GCT CAG GAG ACG TGA         1001
Gly Pro Gly Arg Ala Cys Ala Ala Asp Pro Ala Gln Glu Thr  *
                245                 250                 255

CCCCAGCCCC TGTGGAGGGG GTGCTGGCCC CCAGCCCCGC CCGCCTGGCC CCTGGGCCTG  1061

CCAAGGATGA CACTGCCAGT CACGAGCCCG CTCTGCACAG GGATGACCTG GAGTCCCTTC  1121

TGAGCGAGCA CACCTTCGAT GGCATCCTGC AGTGGGCCAT CCAGAGCATG GCCCGTCCGG  1181

CGGCCCCCTT CCCCTCCTGA CCCCAGATGG CCGGGACATG CAGCTCTGAT GAGAGAGTGC  1241

TGAGAAGGAC ACCTCCTTCC TCAGTCCTGG AAGCCGGCCG GCTGGGATCA AGAAGGGGAC  1301

AGCGCCACCT CTTGTCAGTG CTCGGCTGTA AACAGCTCTG TGTTTCTGGG GACACCAGCC  1361

ATCATGTGCC TGGAAATTAA ACCCTGCCCC ACTTCTCTAC TCTGGAAGTC CCCGGGAGCC  1421

TCTCCTTGCC TGGTGACCTA CTAAAAATAT AAAAATTAGC TG                      1463
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 254 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Met Trp Leu Val Tyr Ser Ser Gly Ala Pro Gly Thr Gln Gln Pro Ala
 1               5                  10                  15

Arg Asn Arg Val Phe Phe Pro Ile Gly Met Ala Pro Gly Gly Val Cys
            20                  25                  30

Trp Arg Pro Asp Gly Trp Gly Thr Gly Gly Gln Gly Arg Ile Ser Gly
        35                  40                  45
```

Pro Gly Ser Met Gly Ala Gly Gln Arg Leu Gly Ser Ser Gly Thr Gln
50 55 60

Arg Cys Cys Trp Gly Ser Cys Phe Gly Lys Glu Val Ala Leu Arg Arg
65 70 75 80

Val Leu His Pro Ser Pro Val Cys Met Gly Val Ser Cys Leu Cys Gln
85 90 95

Lys Asn Glu Asp Glu Cys Ala Val Cys Arg Asp Gly Gly Glu Leu Ile
100 105 110

Cys Cys Asp Gly Cys Pro Arg Ala Phe His Leu Ala Cys Leu Ser Pro
115 120 125

Pro Leu Arg Glu Ile Pro Ser Gly Thr Trp Arg Cys Ser Ser Cys Leu
130 135 140

Gln Ala Thr Val Gln Glu Val Gln Pro Arg Ala Glu Glu Pro Arg Pro
145 150 155 160

Gln Glu Pro Pro Val Glu Thr Pro Leu Pro Pro Gly Leu Arg Ser Ala
165 170 175

Gly Glu Glu Pro Arg Cys Gln Gly Trp Thr Pro Arg Pro Cys Thr Pro
180 185 190

Tyr Cys Val Trp Val Leu Arg Val Ser Arg Thr Trp Leu Leu Val Arg
195 200 205

Val Ala Gly Cys Ala Glu Met Val Arg Thr Cys Cys Gly Val Leu Thr
210 215 220

Ala Pro Leu Pro Ser Thr Gly Ala Ala Thr Ser Gln Pro Ala Pro Pro
225 230 235 240

Gly Pro Gly Arg Ala Cys Ala Ala Asp Pro Ala Gln Glu Thr *
245 250 255

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GATGACACTG CCAGTCACGA 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTTCCCGAGT GGAAGGCGCT GC 22

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AGGGGACAGG CAGGCCAGGT 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GAGTTCAGGT ACCCAGAGAT GCTG 24

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTCGCTCAGA AGGGACTCCA 20

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGATTCAGAC CATGTCAGCT TCA 23

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GTGCTGTTCA AGGACTACAA C 21

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TGGATGAGGA TCCCCTCCAC G 21

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCATCCTAAT ACGACTCACT ATAGGGC 27

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TGCAGGCTGT GGGAACTCCA 20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

AGAAAAAGAG CTGTACCCTG TG 22

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TGCAAGGAAG AGGGGCGTCA GC 22

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TCCACCACAA GCCGAGGAGA T 21

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ACGGGCTCCT CAAACACCAC T 21

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

TGGAGATGGG CAGGCCGCAG GGTG 24

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CAGTCCAGCT GGGCTGAGCA GGTG 24

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GCGGCTCCAA GAAGTGCATC CAGG 24

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CTCCACCCTG CAAGGAAGAG GGGC 24

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Thr Leu His Leu Lys Glu Lys Glu Gly Cys Pro Gln Ala Phe His
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Gly Lys Asn Lys Ala Arg Ser Ser Ser Gly Pro Lys Pro Leu Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

ATGGCGACGG ACGCGGCGCT ACGC

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CCTGGATGTA CTTCTTGGAG CCGC

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GAGCCCGAGG GGCCGTGGAG GGGA

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GGCTGCACCT CCTGGACTGT TGCC

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GATCCTGCTC AGGAGACGTG ACCC

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

CACCAGGCAA GGAGAGGCTC CCGG

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

CCACCCCATG GCGACGGACG

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GGAATTCGGA GGGGAAGGGG GCCGCCGGA

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GGACTGAGGA AGGAGGTGTC CTTC

2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Asp Gly Ile Leu Gln Trp Ala Ile Gln Ser Met Ala Arg Pro Ala Ala Pro Phe Pro Ser
1 5 10 15 20

Claims

1. An isolated DNA sequence characterized by comprising the sequence id. no. 1 or a functional fragment or variant thereof, or an functionally equivalent isolated DNA sequence hybridizable thereto.

2. An isolated DNA sequence according to claim 1, characterized in that it is associated with diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

3. An isolated DNA-sequence according to claim 1 or 2, characterized in that it includes a gene defect responsible for APECED.

4. A DNA sequence according to claim 1, characterized by having the sequence according to sequence id. no 1 or a functional fragment thereof having the sequence according to sequence id. no 3 or sequence id. no 5.

5. A protein characterized by comprising the amino acid sequence id. no. 2 or a functional fragment or variant thereof.

6. A protein according to claim 5, characterized in that it is associated diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

7. A protein according to claim 5 or 6, characterized by having the amino acid sequence id. no. 2, or a functional fragment thereof having the sequence according to sequence id. no. 4, or a functional fragment thereof having the sequence according to sequence id. no 6.

8. A protein according to any of claims 5 to 7, characterized by having distinct structural motifs, including the PHD finger motif (PHD), the LXXLL motif (L), proline-rich region (PRR), and cystein-rich region (CRR).

9. A method for the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, characterized by detecting in a biological specimen the presence of a DNA sequence comprising the sequence id. no. 1 or a functional fragment or variant thereof, or a functionally equivalent isolated DNA-sequence hybridizable thereto.

10. A method according to claim 9, characterized in that the DNA sequence is associated with APECED.

11. A method according to claim 9 or 10, characterized in that the DNA sequence includes a gene defect responsible for APECED.

12. A method according to claim 11, characterized in that the gene defect to be detected includes a "C" to "T" transition resulting in the "Arg" to "Stop" nonsense mutation at amino acid position 257 and/or a "A" to "G" transversion resulting in the "Lys" to "Glu" missense mutation at amino acid position 42.

13. A method according to any one of claims 9 to 12, characterized in that DNA techniques are used for the detection.

14. A method according to any one of claims 9 to 13, characterized in that the detection takes advantage of TaqI or another enzyme cleaving at recognition site 5'-TCGA-3' digestion.

15. A method according to any one of claims 9 to 14, characterized in that the disease is autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

16. A method for the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, characterized by detecting in a biological specimen the presence or the absence of a protein comprising the sequence id. no. 1, or a functional fragment thereof having the sequence according to sequence id. no. 4, or a functional fragment thereof having the sequence according to sequence id. no. 6.

17. A method according to claim 16, characterized in that the protein is associated with APECED.

18. A method according to claim 16 or 17, characterized in that the disease is autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

19. The use of the DNA sequence according to any one of claims 1 to 4 in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

20. The use of the protein according to any one of claims 5 to 7 in the diagnosis of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

21. The use of the DNA sequence according to any one of claims 1 to 4 for the preparation of a medicament useful in a gene therapy method of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

22. The use of the DNA sequence according to any one of claims 1 to 4 in the treatment of diseases related to immune maturation and regulation of immune response towards self and nonself, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).

23. Reagents reacting with the DNA sequence according to any one of claims 1 to 4 or the protein of any one of the claims 5 to 8 or with reagents reacting therewith.

24. Reagents according to claim 23, characterized in that they are antibodies.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00749

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/47, A61K 38/17
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAPLUS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Nature genetics, Volume 17, December 1997, The Finnish-German APECED Consortium, "An autoimmune disease, APECED, caused by mutations in a novel gene featuring two OHD-type zinc-finger domains" page 399 - page 403 --	1-8,19-24
A	Am. J. Hum. Genet., Volume 59, 1996, P. Björses et al, "Genetic Homogeneity of Autoimmune Polyglandular Disease Type I" page 879 - page 886 --	1-8,9-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 January 1999	25 -01- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerlöf Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00749

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Genome Research, Volume 7, August 1997, Johanna Aaltonen et al, "High-Resolution Physical and Transcriptional Mapping of the Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy Locus on Chromosome 21q22.3 by FISH" page 820 - page 829	1-8,19-24

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 98/00749

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 9-18
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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(54) Title: AUTOANTIBODIES IN PATIENTS WITH ACQUIRED HYPOPARATHYROIDISM AND ASSAY METHOD THEREFOR

(57) Abstract

Acquired hypoparathyroidism (AH) occurs frequently as a component of type I autoimmune polyglandular diseases (APS-I) or as a sporadic isolated disease in adults. Whereas autoantibodies to the parathyroid glands have been reported in AH, their very existence remains controversial. We report here the detection of autoantibodies as identified through immunoblotting using sera from AH patients. The antibodies were specific to parathyroid autoantigens obtained through differential centrifugation of fresh human hypercellular parathyroid glands, obtained shortly after their surgical removal for tertiary hyperparathyroidism in patients with renal failure. Autoantibodies were detected against several parathyroid specific proteins in the 17 AH sera tested. Twelve (70 %) reacted to a protein of 70 kDa, while 16 (94 %) reacted to a protein of 80 kDa, and 3 (18 %) reacted to a protein of 120-140 kDa, respectively. The 70 and 80 kDa proteins were localized to the cytosolic fraction of the parathyroid extract, and the 120-140 kDa protein to the membrane fraction. The autoantibodies were also detectable using a dog parathyroid gland as an antigen source. However, they could not be identified using cultured human melanocytes or in rat pituitary cells as antigen sources. Sera from 50 patients with various other autoimmune diseases as well as 22 normal controls were also tested, and none reacted with any of the above specific parathyroid proteins. It is concluded that autoantibodies to 3 major autoantigens in the human parathyroid gland extract have been detected in AH patients. Furthermore, our data indicates that the 120-140 kDa autoantigen is the calcium sensing receptor and that the reactive epitope largely resides in its extracellular domain. These data confirm the autoimmune nature of AH, and the invention provides a method for detecting AH or propensity for AH as well as a method for treating AH.

DESCRIPTION

AUTOANTIBODIES IN PATIENTS WITH ACQUIRED HYPOPARATHYROIDISM AND ASSAY METHOD THEREFOR

The work associated with this invention was supported by grants from Genetech Foundation for Growth and Development (94-338), the American Diabetes Association (Mentor Award), and National Institutes of Health (RO1 HD 19469). The government has certain rights in this invention.

Background of the Invention

i. Field of the Invention

This invention relates to autoantibodies associated with acquired hypoparathyroidism, the autoantigens recognized by these autoantibodies, and to assay methods useful in the detection and treatment of acquired hypoparathyroidism.

ii. Background

Acquired hypoparathyroidism (AH) results from a deficiency of parathyroid hormone (PTH) secretion which leads to hypocalcemia. PTH plays an important role in calcium homeostasis, in that it can enhance calcium resorption from bone, increase calcium reabsorption from the kidney, and act on the renal tubule to promote the conversion of 25-hydroxyvitamin D to its active 1,25-dihydroxy metabolite, which in turn, increases the intestinal calcium absorption (Conklin, 1994; Lund, 1980). The most prominent clinical features of hypocalcemia are muscular irritability, tetany and seizures (Aurbach, 1990).

AH occurs as a common component of type I autoimmune polyglandular syndrome (APS I) which presents in infants or young children. AH also occurs in association with Hashimoto thyroiditis and/or hypothyrodism, a combination which most often affects adult women. APS I is characterized by mucocutaneous candidiasis, hypoparathyroidism and Addison disease, often accompanied by early onset pernicious anemia, chronic active hepatitis, alopecia and primary hypogonadism (Neufeld, 1980; Neufeld, 1981; Winter, 1992, Maclaren, 1985; Muir, 1991). APS I occurs either as an autosomal recessive disease, or as a seemingly sporadic disorder. Whereas APS I is not linked to genes within the HLA-DR region (Ahonen, 1988), the responsible gene has been recently mapped to chromosome 21q22.3 (Aaltonen, 1994). AH may result in mental retardation or unexplained epilepsy if it is not appropriately recognized and treated by the relatively simple and inexpensive expedient of activated vitamin D therapy and calcium

supplementation. In APS I, however, normalization of calcium levels through vitamin D supplements may be confounded by malabsorption.

An autoimmune etiology for AH has been suggested because of its association with other autoimmune diseases (Ahonen, 1990), and by reports of autoantibodies directed against the parathyroid tissues in affected individuals. Autoantibodies to the parathyroid glands were first reported by Blizzard et al (Blizzard, 1966). In that study, 38% of 74 patients with autoimmune hypoparathyroidism were found to be positive compared with only 6% of 245 healthy control subjects. The results from subsequent studies were controversial, since the antibodies often appeared to be directed against mitochondrial antigens. These may be false positive results, because mitochondrial rich cells are found within normal parathyroid tissue and anti-mitochondrial antibodies are common in autoimmune disorders (Betterle, 1985). Autoantibodies from the sera of patients with sporadic adult onset hypoparathyroidism however have been reported to bind to the cell surfaces of human parathyroid cells, resulting in an inhibition of PTH secretion (Posillico, 1986). In addition, autoantibodies in the sera of patients with AH have been reported to be cytotoxic for cultured bovine parathyroid cells, by an antibody mediated cytotoxicity dependent on complement fixation and activation (Brandi, 1986; Fattorossi, 1988). Whereas the above findings may suggest the possible involvement of autoantibodies against parathyroid glands in AH, the nature of the targeted autoantigens has only recently been identified by Song *et al.*, Abstract No. OR2-3, 77th Annual Meeting of the Endocrine Society, Washington, D.C., June 14-17, 1995.

The regulation of PTH secretion requires that parathyroid cells sense the serum free ionized calcium levels in extracellular fluid. The recently cloned calcium sensing receptor (CA-SR) responds to increased levels of extracellular calcium by triggering a phospholipase-C (PLC) dependent pathway which in turn induces the parathyroid cell to decrease its constituent PTH secretion (Conklin, 1994). The CA-SR gene has been mapped to chromosome 3q2 (Pollak, 1993), and the translated protein is a linear peptide of 1085 amino acids, with 7 putative transmembrane-spanning domains and a large extracellular domain of 613 amino acids at the amino terminus (Brown, 1993). The external domain probably serves as the actual ionized calcium detector. It is a member of a new family of G-protein-coupled receptors which is densely expressed in parathyroid cells, but to some extent also in thyroid C-cells, brain and kidney. Mutations of the CA-SR gene have been found to be responsible for familial benign hypocalciuric hypercalcemia (FBHH) and autosomal dominant hypocalcemia (Pollak, 1993).

The following references are discussed in greater detail below:

1. Betterle, C., *et al.* (1985) *Clin. Exp. Immunol.* 62:353-360.
2. Blizzard, R.M., *et al.* (1966) *Clin. Exp. Immunol.* 1:119-128.
3. Brandi, E.M., *et al.* (1986) *P.N.A.S. USA* 83:8366-8369.
4. Brown, E.M., *et al.* (1993) *Nature* 366:575-580.

5. Fattorossi, A., *et al.* (1988) *P.N.A.S. USA* 85:4015-4019.

6. Irvine, W.J., *et al.* (1969) *Clin. Exp. Immunol.* 4:505-510.

7. Juhlin, C., *et al.* (1987) *P.N.A.S. USA* 84:2990-2994.

None of these references teach or suggest that the autoantibodies detected in patients with AH have, as their antigenic target, the calcium sensing receptor expressed by parathyroid chief cells. In addition, none of these references make any mention of the 70 or 80 kilodalton proteins which are part of the instant invention.

The Blizzard reference noted the existence of antibodies in patients with hypoparathyroidism which seemed to react in an organ specific manner with parathyroid tissue. The reactive antigen was not identified, and the authors cast doubt on whether such autoantibodies could have any etiologic relationship to the development of AH. Thus, Blizzard *et al.* teach away from the instant invention which establishes such a relationship. It is noted, however, that Blizzard *et al.* make reference to publications of Davis, *et al.* (Lancet ii, 1432, 1961) and of Jones & Fourman (Lancet ii, 119, 1963; Lancet ii, 121, 1963), in support of the suggestion that "a test to detect subclinical hypoparathyroidism ... would permit detection of partial hypoparathyroidism." The assay method of the instant invention is different from the test taught in those references.

The Irvine reference reported the presence of antibody which reacted with both parathyroid oxyphil and chief cells in a patient with idiopathic hypoparathyroidism. However, the identity of the antigens involved in those immune responses was not determined.

The Betterle reference suggests the existence of anti-mitochondrial autoantibodies in idiopathic hypoparathyroidism, and takes exception to Blizzard *et al.*'s suggestion that the autoantibodies detected in AH are parathyroid organ specific. The antigen identified by Betterle *et al.* was a 46 kilodalton protein, clearly different from the antigens identified in the instant invention.

The Brandi *et al.* reference discloses an antibody in AH patients which was cytotoxic for parathyroid and adrenal cortex cells, but not for pituitary, thyroid, liver or kidney cells. The assay method disclosed depends on cell culture and cytolysis (release of ^{51}Cr) or immunofluorescence. The antigens involved in the method are not identified. Thus, a specific antigen based assay method is neither disclosed nor suggested.

The Juhlin reference relates to the development of monoclonal antibodies against whole parathyroid cells which exclusively recognize antigens on parathyroid and kidney tubule cells. The authors speculate that the antibodies bind to and block a cell surface calcium sensing receptor on these cells. However, the antibodies used in the study were not autoantibodies, and the nature of the antigen recognized was not defined, other than speculatively, based on the functional changes observed upon antibody binding.

The Fattorossi reference is a follow-up and an extension of the work reported by Brandi *et al.* The cytotoxic antibody was further classified as an IgM, it was found to not be organ specific, it reacted with endothelial cells, and these researchers found that two target antigens, one of molecular weight 200 kilodaltons and the other of 130 kilodaltons, were recognized. There is no suggestion that the target antigens are related to the parathyroid specific calcium sensing receptor.

The Brown reference discloses the cloning of a bovine parathyroid extracellular calcium sensing receptor having a molecular weight of about 120 kilodaltons. There is no suggestion that there is any relationship between this receptor and autoimmune hypoparathyroidism.

Several patent publications are also discussed below:

1. US Patents 5,053,491 and 4,864,020, issued to Cance *et al.*, Washington University:

These patents relate to monoclonal antibodies specific for an antigen present on the surface of parathyroid tissue and are used to identify parathyroid tissue. The antigen is a 191 kilodalton protein (unreduced; reduced molecular weight is about 171 kilodaltons). By contrast, the instant method and compositions relate to antigens of 120 kD, 70 kD and 80 kD; and fragments thereof.

2. WO 88/03271 and related foreign equivalents, Juhlin *et al.*:

This patent publication is related to the above noted Juhlin scientific publication discussed above.

3. WO 94/28019 and related foreign equivalents, Juhlin *et al.*:

This patent publication relates to the work of Brown *et al.*, discussed above, which disclosed cloning of the bovine calcium sensing receptor. This patent application relates to the cloning of the human calcium sensor, which they refer to as the calcium sensor protein, or CSP. The CSP clone is said to be useful for obtaining agonists/antagonists to the CSP and for obtaining antibodies to the CSP. The antibodies and agonists/antagonists so obtained are predicted to be useful in the treatment of a number of disease states including hyperparathyroidism. Thus, like the Brown *et al.* publication, this publication does not make any connection between the CSP and autoimmune hypoparathyroidism. Of interest is the identification of this molecule by the WO 94/28019 applicants as having a molecular weight of 500 kDa. The instant invention, by contrast, relates to the human parathyroid calcium sensing receptor as a 120 kDa molecule.

4. US Application No. 07/356,999, US Department of Health and Human Services, Aurbach:

This patent publication relates to the above discussed Brandi *et al.* and the subsequent Fattorossi *et al.* publications. This publication refers to a bovine parathyroid cell line in culture which, it is urged, is useful for the characterization of the antibody in autoimmune hypoparathyroidism. A specific antigen based assay method is neither taught nor suggested.

In the paragraph bridging pages 17 and 18 of the reference, it is postulated that the availability of a clone for the 500 kDa protein may assist in studies of autoimmune hypoparathyroidism. A publication of Brown, E. M. (1991) *Phys. Rev.* 71, 371-411 is cited as implicating autoimmunity in the pathogenesis of rare idiopathic hypoparathyroidism.

By contrast of the above discussed references this invention documents the existence of parathyroid autoantibodies in AH, and characterizes the reacting human specific parathyroid autoantigens. The parathyroid reactive autoantibodies are frequent in AH, and the CA-SR is an important autoantigen target in the disease.

Our finding that parathyroid cell derived CA-SR is a major autoantigen in AH targeted by autoantibodies is a novel observation, adding to the list of autoantigens that are receptors targeted by an immune response in an organ specific autoimmune disease (Wilkin, 1990). The thyroid stimulating hormone (TSH) receptors in autoimmune thyroid disease (Davies, 1981), acetylcholine (ACh) receptors of skeletal muscle in myasthenia gravis (Gonzalez-Ros, 1984; Fumagalli, 1982; and Aesonki, 1981), gastrin receptors in pernicious anemia (Loveridge, 1980), corticotropin receptors in Addison disease (Kendall-Taylor, 1988), and insulin receptors in IDD (Maron, 1983) are important autoantigens to their respective diseases. The mechanisms for involvement of receptors in autoimmune responses are probably complex. The first possibility is that autoantibodies against cell-surface receptors may lead to functional abnormalities of the cells expressing them, resulting in receptor-mediated stimulation or inhibition of the targeted cells. One example of stimulation by an agonist autoantibody mimicking a physiologic molecule is Graves disease which is caused by the binding of autoantibody to TSH receptors such that they are stimulated but in a more prolonged manner than for TSH itself (long acting thyroid stimulator or LATS). One example of inhibition by antagonist autoantibody is myasthenia gravis which is caused by the binding of antibody to ACh receptors. The second possibility is that the autoantibodies cross-link the receptors and increase the rate of their degradation which ultimately lead to their depletion. The third possibility is that the autoantibody can bind to the receptor, fix complement, and thereby induce damage to the cells expressing the receptor. In our study, the autoantibody directed against the external domain of the CA-SR may play a direct role in the pathogenesis of AH as an agonist autoantibody. The autoantibody could induce the same effect as normal serum ionized calcium by binding and activating the receptors which in turn stimulate signal transduction events thereby raising intracellular ionized calcium levels and depressing PTH secretion. That we found the external domain and not the internal domain of the CA-SR to contain the reactive epitope of parathyroid autoantibodies in AH, suggesting a functional role for

the autoantibodies. This is also supported by a previous report that autoantibodies from the sera of patients with adult-onset AH bound to the cell surface of human parathyroid cells and induced inhibition of PTH secretion (Posillico, 1986). Alternatively, the autoantibodies could directly induce damage to the PTH-secreting cells. This is consistent with other reports that autoantibodies in the sera of patients with AH were found to be cytotoxic to cultured bovine parathyroid cells by an antibody mediated cytotoxicity (Brandi, 1986; Fattorossi, 1988), and that the epithelial cells of the parathyroid glands in AH patients were either diminished in number or absent by histological studies, sometimes accompanied by a lymphocytic infiltrate. T cells may be contributors to the pathogenesis of AH (Wortsman, 1992), however, we conclude that AH may be predominantly an antibody-mediated disease for which the instant detection and treatment method provides a significant solution.

Brief Summary of the Invention

Acquired hypoparathyroidism (AH) has been considered to result from an autoimmune process, but the targeted self-antigens have only recently been identified. This invention discloses our study which included 25 patients with AH, of which 17 had type I autoimmune polyglandular syndrome and 8 were associated with autoimmune hypothyroidism. Autoantibodies were sought against antigens in hyperplastic human parathyroid glands using an immunoblotting assay. We discovered that twelve of 17 (70%) AH sera that we tested reacted to a cytosolic antigen of 70 kDa, and 16 (94%) to one of 80 kDa. Four of 20 (20%) AH sera also reacted to a membrane associated antigen of 120-140 kDa, the exact size of the calcium sensing receptor (CA-SR) dependent upon its degrees of glycosylation. None of the sera from 50 patients with autoimmune diseases other than AH or 22 normal disease-free controls had such parathyroid autoantibodies. Six of 20 (30%) sera from AH patients reacted to the CA-SR transfected on HEK-293 cells while 14 of all 25 (56%) AH sera reacted to external domain (60-70 kDa) of the CA-SR translated *in vitro* by a rabbit reticulocyte expression system. These discoveries confirm the role of the antigens in the pathogenesis of AH.

The findings of parathyroid specific autoantibodies in many patients with AH document the autoimmune nature of this disease, while the localization of the reactive epitope of the CA-SR to its external domain suggests that activation of the receptor could induce inhibition of PTH secretion in the disease.

This invention is a method useful in the detection of autoantibodies in persons that have developed, or are at risk of developing AH. The specific targets of autoantibodies associated with this disease are identified as: (1) the extracellular portion of the calcium sensing receptor which has a total molecular weight of about 120-140 kilodaltons, expressed on the surface of chief cells of the parathyroid; and (2) two additional, cytosolic antigens, one having a molecular weight of about 70 kilodaltons, and the other having a molecular weight of about 80 kilodaltons. It is noted

that a higher percentage of AH antisera react with the 70 or 80 kilodalton proteins than with the 120-140 kilodalton receptor protein. One utility of this invention is that the identified autoantigens, or antigenic fragments thereof, may be used to achieve early diagnosis and treatment of AH, and possibly other autoimmune diseases which are frequently associated with AH.

Accordingly, one object of this invention is to provide a method for detecting autoimmune hypoparathyroidism in a patient, or propensity for developing autoimmune hypoparathyroidism and the propensity to develop other autoimmune diseases which are commonly associated with development of autoimmune hypoparathyroidism.

Another object of this invention is to provide a method for detecting autoimmune hypoparathyroidism related autoantigens.

Another object of this invention is to provide a method for obtaining isolated polynucleotide which encodes an autoimmune hypoparathyroidism related autoantigen.

Another object of this invention is to provide a method for making a recombinant cell transfected with an isolated polynucleotide which encodes an autoimmune hypoparathyroidism related autoantigen.

Another object of this invention is to provide a method for treating patients with autoimmune hypoparathyroidism or those at risk of developing autoimmune hypoparathyroidism.

Brief Summary of the Figures

Figure 1 – Immunoblot analysis using membranes of HEK-293 cells transfected with human CA-SR cDNA. Immobilon strips containing the antigens were incubated with IgG of rabbit anti-CA-SR Ab (lane 1), AH patient sera (lane 2) and normal human sera (lane 3).

Figure 2 – Immunoprecipitation of the in vitro translated external domain of the CA-SR. In vitro translated products were incubated with AH patient sera (lane 1 - lane 3) and normal human sera (lane 4 - lane 6).

Detailed Description of the Invention

As the examples which follow demonstrate, our studies confirm the autoimmune nature of acquired hypoparathyroidism (AH) and demonstrate that autoantibodies in patients with AH target human parathyroid proteins of 70, 80 and 120-140 kDa. The autoantigens are disease-specific since they were only recognized by the sera from patients with AH and not from those with other autoimmune diseases. In addition, the autoantigens are tissue-specific since they were not detected by immunoblotting using cultured human melanocytes or rat pituitary cells as antigen sources. The autoantigens are not mitochondrial proteins, because we were careful to remove the mitochondrial proteins by centrifugation prior to immunoblotting in our studies. Furthermore, we chose fresh human hypercellular parathyroid glands and normal dog parathyroid glands instead of parathyroid cell lines or tumors, so that we could avoid spurious identification of any antigens

that are not normally present in the parathyroid glands. The 120-140 kDa protein is the calcium sensing receptor CA-SR.

Using an immunoblotting assay and sera from 25 patients with AH, we discovered that 12/17 (70%) of the sera reacted with a 70 kDa antigen in the cytosol of the parathyroid, while 16/17 (94%) reacted with an 80 kDa antigen in the cytosol of the parathyroid. Four out of twenty sera (20%) reacted with a 120-140 kDa membrane associated antigen which we confirm to be the calcium sensing receptor or CA-SR, by reaction of these sera with cloned and expressed CA-SR.

Of interest, most of the CA-SR autoantibody positive patients (including 4 AH in the context of APS I and all 8 adult-onset AH in association with thyroid disease) were females. This finding is consistent with findings in other autoantibody mediated receptor-targeted diseases which occur predominantly in females. Four of our adult-onset AH patients developed their disease and had the CA-SR autoantibodies detected after they had babies, another 2 adult-onset AH patients developed their disease after menopause, while one who presented with AH in the context of APS I began her disease at the onset of her menses. This suggests a possible influence of female hormones in the etiology of the disease.

That autoantibodies to CA-SR were absent from some AH in the context of APS I patients could be explained by the complete loss of the autoantigen needed to drive their formation before they could be studied. Two of the CA-SR autoantibody negative AH patients had developed their disease 32 years prior to the study, while another 2 autoantibody negative AH patients developed their diseases more than 10 years prior to the study. A general characteristic of all autoimmune diseases is that there are remissions and exacerbations of the underlying pathogenic processes involved over time. With insulin dependent diabetes (IDD), islet cell autoantibodies (ICA) disappear following clinical onset of disease when the pancreatic ß cells are destroyed, and, as a result, the ICA reactive self antigens have disappeared. AH may be no exception. Thus, some of our patients affected by AH undoubtedly had CA-SR autoantibodies at an earlier time point in their disease but had lost them before we could study them. Finally, although the Western blot technique can be used and is specific for antigen reactivity, it is a relatively insensitive method for detecting autoantibodies. Other more sensitive assays, such as radioimmunoassay or ELISA, are able to increase the autoantibody detection frequencies (Song, 1994), and are therefore preferred.

Interestingly, 8 adult-onset AH patients in our study were associated with autoimmune hypothyroidism. Since the CA-SR transcripts have also been detected in the thyroid glands by Northern blots (Brown, 1993), an autoimmunity directed to the CA-SR could account for the involvement of both organs. Thus, the detection method and treatment methods disclosed herein are applicable to AH as well as hypothyroid related pathologies. One male AH in the context of APS I had high titers of CA-SR autoantibody in addition to thyroid microsomal and thyroid thyroglobulin autoantibodies. In contrast, the autoantibodies to CA-SR were not detected from

the 8 sera from age, sex and ethnicity matched control patients with hypothyroidism alone. Therefore, additional local events may be required to allow the development of an immune response to the CA-SR. Furthermore, the autoantibodies to CA-SRs on the parathyroid gland could be the initial phenomenon leading to PTH deficiency, followed by autoantibody reactivity to the CA-SRs on the thyroid glands and their subsequent involution through an immune mediated destruction.

The CA-SR appeared as 120-140 kDa bands on our immunoblots, and the external domain of the CA-SR appeared as 60-70 kDa bands on immunoprecipitation, due to differential glycosylation of the receptor components. However, this differential glycosylation did not appear to affect the antigenic structure of the CA-SR since both the 120-140 kDa and 60-70 kDa bands were well recognized by both the rabbit antibody and the AH patient sera. HEK-293 cells transfected with CA-SR contain more CA-SRs than normal human parathyroid gland membrane preparations. This may explain why more of the AH patients we studied were found to be positive when CA-SR transfected HEK-293 membranes, rather than human parathyroid gland membranes, were used as antigen sources in immunoblotting. In addition, we found that the *in vitro* translated external domain of CA-SR had much less background than the transfected HEK-293 cells and this may explain why as many as 56% of the AH patients we tested, many of whom had long-standing AH, were found to be positive when *in vitro* translated external domain of CA-SR was used as the antigen source in immunoprecipitation studies.

We have confirmed that the 70 and 80 kDa parathyroid protein antigens are not related to parathyroid secretory protein (PSP), which has a molecular weight of 70 kDa and is co-stored and co-secreted with PTH in the parathyroid gland (Leiser, 1989). That protein has been shown to be present in the secretory granules of a variety of neuroendocrine and endocrine tissues with greatest abundance in parathyroid glands and adrenal medulla. However, in tests using AH positive sera, PSP produced using an *in vitro* translation based immunoprecipitation technique revealed no positive reactivities.

In view of the above disclosure and the examples and further disclosure which follows, it will be obvious to those skilled in the art that our finding of autoantibodies to the CA-SR and to the 70 and 80 kDa parathyroid specific antigens in AH patients give rise to a valuable diagnostic test for the disease. Positive test results in the diagnostic test, described in detail below, indicates the need to initiate a regimen of compensatory calcium, activated vitamin D therapy, immunotherapy, or a combination of these therapies.

The diagnostic test of this invention is a method for detecting autoimmune hypoparathyroidism in a patient, or the propensity for developing autoimmune hypoparathyroidism and the propensity to develop other autoimmune diseases which are commonly associated with development of autoimmune hypoparathyroidism, such as hypothyroidism. The method comprises detecting the presence of serum antibodies which react

with the human calcium sensing receptor (CA-SR), or antigenic subportions thereof, or which react with parathyroid specific cytosolic antigens which are associated with autoimmune hypoparathyroidism.

The method may use the entire CA-SR or merely the antigenic subportion of the human calcium sensing receptor, such as the external domain of the receptor. A source of parathyroid cell membranes expressing the CA-SR, or a membrane preparation from recombinant cells expressing the CA-SR is obtained according to methods well known in the art using the known sequence of the CA-SR (see materials and methods below) and may be used in the method.

Alternatively, or in addition, the method may utilize the purified or cloned, expressed and purified 70 kilodalton or 80 kilodalton cytosolic antigen present in parathyroid cells. These proteins are easily purified according to methods well known in the art, such as by preparing an affinity column to which is immobilized antiserum from an AH patient which is known to react with these cytosolic proteins. By preparing another column from an AH negative patient's serum, all proteins other than the 70 kDa or 80 kDa proteins may be adsorbed out of a cytosolic parathyroid preparation. The flow-through of this first column is then passed over the AH serum column, and the bound, purified antigens eluted by reducing pH or by other means well known in the art of immunoaffinity chromatography. As a final polishing step and to separate the 70 kDa from the 80 kDa protein, a size exclusion column or reversed phase HPLC column is run. The resulting isolated antigens may be used directly in the diagnostic method of this invention.

Alternatively, according to methods well known in the art, the proteins may be subjected to amino acid analysis and the amino acid sequence thereby derived used to design oligonucleotide probes for cloning the genes encoding these proteins. This is accomplished by screening either a human genomic library, or a human parathyroid cDNA library, both of which are commercially available or which may be prepared according to methods well known in the art (see Maniatis et al., Cold Spring Harbor, *Molecular Cloning*, 1982). Once identified, clones of the 70 and 80 kilodalton encoding nucleic acid sequences are isolated by restriction digestion and subcloned into an expression vector appropriate for expression of the encoded antigens in any given recombinant cell of choice, including but not limited to bacterial cells, yeast cells and mammalian cells. Those skilled in the art are well aware of the need to provide appropriate transcriptional initiation and termination signals and the need to provide appropriate translation start and stop codons. In addition, the nucleic acids encoding the 70 or 80 kDa parathyroid cytosolic antigens may be modified to adapt the coding sequences for optimal expression in the cell type chosen, such as by optimization of codon usage to match that of highly expressed proteins in that cell type.

Once expressed, the recombinant proteins may be recovered and isolated in essentially the same manner described above for the isolation of the naturally occurring antigens, with minor modifications based on the specifics of the recombinant cell type in which the antigen was expressed. The isolated antigens may then be used to generate a supply of antibodies, including

monoclonal antibodies, according to known methods, which recognize the 70 or 80 kilodalton proteins. These antibodies may be used, for example, in the assay method described below. The 70 and 80 kDa antigens may also be labeled, with a radioactive tag, a fluorescent tag, an enzyme such as horse radish peroxidase, with avidin, with biotin, or any of a number of other known labels, such that competition experiments may be performed using the labeled antigens.

Once an adequate source of CA-SR rich membranes or antigenic portions of the CA-SR and/or 70 and 80 kDa parathyroid antigens is assured, either by isolation of the natural proteins or by recombinant expression as described above, the diagnostic test of this invention may be performed as follows.

Serum is isolated from a patient suspected of having autoimmune hypoparathyroidism or for whom it is important to rule out this possible condition.

The serum is reacted with membranes rich in CA-SR or with the isolated 70 and 80 kDa proteins and the interaction is quantitated by any of a number of methods known in the art. Thus, for example, the presence of AH specific autoantibodies is detected by contacting the patients' serum with biotin labeled AH antigen (70 kDa protein, 80 kDa protein, CA-SR membranes, or antigenic fragments thereof). The reaction is then contacted with a bead coated with immobilized avidin which reacts with the biotin on the AH antigen. If any antigen-antibody complex is formed, this is detected by contacting the beads with anti-human antiserum having a detectable label, such as a radioactive, fluorescent, enzyme or other moiety.

A similar detection assay may be run using CA-SR expressing membranes by, for example, first immobilizing the membranes (for example on the surface of a microtiter plate), contacting the membranes with test serum, and then contacting the reaction with anti-human antiserum which is labeled as described above.

In addition to the above examples, the detection method may take the form of a western blot assay in which immobilized antigen is reacted with test serum, which is then detected by contacting with labeled antiserum.

From the foregoing disclosure and experimental results it is evident that the CA-SR, the 70 kDa and 80 kDa proteins are major antigens in the pathogenesis of AH, and are thus valuable in the diagnosis and therapy of the disease. Autoantibodies to these proteins are useful in disease prediction both in non-AH relatives of patients affected by AH, as well as in the general population. Such antibodies will be detectable by radioimmunoassay using the isolated or recombinant proteins, peptides or antigenic fragments thereof, by depletion or D-ELISA and/or by ELISA or immunoprecipitation as outlined here. Baculo-viral based eukaryotic expression systems are likely to be preferred, to fold the proteins appropriately, and glycosylate the protein if this enhances the antibody binding ability. However, such expression systems as COS cells, yeast cells, and bacterial cells such as *E. coli* could be used for this purpose as those skilled in the art are readily able to appreciate.

Fluid-based immunoassays using the antigens and antibodies of this invention provide the greatest sensitivity to the method since autoantibodies of relevance mostly react to their respective autoantigen through conformational rather than through linear epitopes. The RIA and D-ELISA methods are most useful in fulfilling these properties. Accordingly, the autoantigens identified herein can provide a chemically based assay giving greater precision, reproducibility, and specificity to identification of AH and susceptibility to AH.

In addition to providing the detection methods described above, our identification of the AH antigens provides a method for antigen mediated immunotherapy based upon the use of recombinant protein antigen as a therapeutic agent to restore immune tolerance in AH. Thus, a method of treating patients with autoimmune hypoparathyroidism or those at risk of developing autoimmune hypoparathyroidism which comprises:

(a) detecting the disease or propensity of developing the disease by screening the patient's serum for autoantibody to a 120-140 kDa antigen, the 70 kDa antigen, the 80 kDa antigen, or a combination of these antigens;

(b) treating patients positive in the test of step (a) either with

(i) compensatory PTH therapy,

(ii) antibodies raised against the auto-antibodies that recognize the antigens used in step (a),

(iii) antigens of step (a) to restore immune tolerance to AH associated antigens, or

(iv) a combination of treatments (i) - (iii).

Cellular responses, such as by proliferation or by cytokine elaboration after *in vitro* exposure to the AH autoantigens are also useful in disease prediction and treatment.

The autoantigen peptide derivatives thereof are used in antigen based therapies, including giving the antigens intravenously to induce anergy; deliberately immunizing against the antigen such as to induce an antibody response mediated by T helper-2 type lymphocytes to induce immunosuppressive effects on the pathogenic T helper-1 lymphocyte subset; or orally fed antigen such as to induce anergy and suppressive effects. In the field of insulin dependent diabetes (IDD), intravenous GAD_{65} antigens have been given in early life in NOD mice and shown to induce reductions in the degree of the inflammatory infiltrates or insulitis lesions and prevent the onset of hyperglycemia (Kauffman *et al.* Nature 1994:366:69-72). Subcutaneous immunizations by insulin and insulin B chains in incomplete Freunds adjuvant will prevent diabetes in NOD mice for prolonged periods without reductions in the insulitis lesions. The infiltrating cells however change their phenotype from ones that make large amounts of interferon gamma to ones that do not. Transfer of splenic lymphocytes from mice protected from diabetes in this way also convey protection for periods of up to a month. The intervention thus induces an active immunosuppressive effect and an insulitis lesion that is protective rather than destructive associated with a switch from Th1 to Th2 responses. Further, the effect must be beyond that

merely involving autoimmunity to insulin, since beta cell destruction is arrested. The release of protective cytokines into the milieux of the islet must then also inhibit adjacent autoimmunity responses to other self-antigens through a bystander effect (Muir, Maclaren *et al.* J Clin Invest 1995:95: 628-634; Ramiya, Muir and Maclaren, Clin immunotherapy 1995:3:177-183.) Repeated feedings of defined autoantigens may also be used to inhibit ongoing autoimmune diseases. In the case of the NOD mice, this has occurred through orally administered insulin (Weiner *et al.* Natl Acad Sci USA 1991:88:10252-10256) as well as through the use of oral feedings of insulin and GAD (Muir, Maclaren *et al.* Diabetes/Metabolism Reviews 1994:9: 279-287). Accordingly, therapeutic methods employing AH autoantigens alone or in combination with other antigens provides a novel therapeutic approach to the treatment of AH which is expected to respond similarly to such treatment as outlined for the IDD studies noted above.

It should be noted that patients with other autoimmune diseases should be considered at-risk of developing or having AH, and should therefore be screened for AH autoantibody according to the methods described herein. Those patients found to be positive for the autoantibody, and their family members, should be considered for treatment according to the methods described herein. The reliable immunological assay of this invention can be automated or developed as a finger stick screening test. Furthermore, this invention provides a kit for detecting AH in a patient or in people at-risk of developing AH. The kit would contain any one or all of the following antigens: the CA-SR or antigenic subportions thereof, the 70 kDa protein, the 80 kDa protein. In addition, provision of animal anti-human immunoglobulin, especially if labeled, would allow immobilized antigen to be bound by AH autoantibody and then be detected by contacting the labeled animal anti-human immunoglobulin with the antigen-autoantibody complex.

Materials and Methods

a. Patients:

We examined sera from 25 patients with AH. Of these, 17 patients had APS I (all of them had AH, 14 had mucocutaneous candidiasis, 10 had Addison disease, many had associated vitiligo, alopecia, chronic active hepatitis and/or primary hypogonadism). Another 8 patients had adult-onset hypoparathyroidism associated with goiter and autoimmune hypothyroidism, confirmed by the presence of thyroid microsomal antibody and/or thyroglobulin antibody (Table 1):

Table 1. Characteristics Of AH Patients

Subject	Number	Gender	Age of Onset (range)
AH in APS I	17	10F & 7M	1 yr - 12 yr
AH in Adult	8	8F & 0M	31 yr - 53 yr

In addition, 8 age-matched and sex-matched adult-onset hypothyroidism patients who had normal calcium levels and no evidence of AH were used as controls. We also studied sera from 10 patients with Addison disease, 10 with Graves disease, 12 with Hashimoto thyroiditis, 10 with insulin dependent diabetes (IDD) and 8 with vitiligo (none of whom had AH), as well as 22 normal disease-free controls. No normal controls had any endocrine-associated serum autoantibodies, such as thyroid microsomal, thyroglobulin autoantibodies, or islet cell autoantibodies.

b. Antigen Preparation

Hypercellular human parathyroid glands were obtained after their surgical removal from three patients being treated for hyperparathyroidism and osteomalacia, associated with renal failure. The parathyroid glands were placed on ice in phosphate-buffered saline (PBS,pH 7.4) with a mixture of protease inhibitors (1,10-phenanthroline, aprotinin, EDTA and benzamidine). The tissue was homogenized with a glass tissue grinder and centrifuged at 15,000 xg to remove cell debris, nuclei and mitochondrial proteins. The supernatants were further separated into membrane and cytosolic fractions by ultracentrifugation at 100,000 xg. Both the membrane and cytosolic fractions were used as antigen sources in immunoblot experiments.

Plasma membrane preparations from HEK-293 cells expressing the CA-SR together with membrane preparations from wild type HEK-293 cells were used as antigen sources in the immunoblotting and immunoprecipitation studies below.

c. Immunoblotting

The cytosolic and membrane fractions were solubilized in sodium dodecyl sulfate (SDS) gel loading buffer containing DL-dithiothreitol (DTT) and heated for 3 minutes at 100^0 C before loading. After separation by SDS-PAGE, the proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA). The strips of the membrane were cut and incubated with 1% BSA in Tris-buffered saline and 0.05% Tween-20 (TBST) to block free potential binding sites. Test sera at 1/100 dilutions as well as purified IgG of a rabbit anti-CA-SR antisera and IgG of

pre-immune rabbit sera were incubated with the antigen-containing strips. The strips were then incubated with an anti-human or anti-rabbit polyvalent immunoglobulin alkaline phosphatase conjugate, and developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Promega, Madison, WI).

d. *In vitro* translation and immunoprecipitation

The CA-SR is a linear peptide of 1085 amino acids, with 7 putative transmembrane domains and a large extracellular domain of 613 amino acids at the amino terminus. The human CA-SR cDNA (Brown, 1993; Pollak, 1993) external and internal domains were amplified by polymerase chain reaction (PCR). The PCR products were positioned downstream of the SP6 promoter on the pcDNA3 construct. The recombinant plasmid was propagated in E. coli and purified by the Magic™ Minipreps System (Promega). The CA-SR cDNA was transcribed and translated as described according to the manufacturer's instructions. In brief, 1 μg circular plasmid DNA was transcribed in a 100 μl reaction for 2 hours at 40^0 C, using SP6 RNA polymerase (Stratagene, La Jolla, CA) in the presence of RNAsin (Promega). The translation was done using a methionine-free rabbit reticulocyte lysate in a 50 μl reaction using 20% of the synthesized RNA as a substrate in the presence of 4 μl ^{35}S-methionine (10 mCi/ml) (Amersham, Arlington Heights, IL).

Once the translation reaction was complete, the translated products were examined by taking 5 μl aliquots mixed with 20 μl of SDS sample buffer. The samples were heated at 100^0 C for 2 minutes and loaded to SDS-polyacrylamide gel (SDS-PAGE). For autoradiography, the gels were dried and exposed to X-ray film (XAR-2 ready pack, Sigma, St Louis, MO) overnight at room temperature. Since CA-SR is a glycoprotein, 1 μl of canine pancreatic microsomes (Promega) were added to the translation reaction mixture in order to obtain the mature glycosylated receptor.

For characterization of the autoantibody reactivities, 1 μl of the translated products were incubated with 2 μl of sera at 4^0 C overnight. The immunocomplexes were washed three times with ice-cold PBS and incubated with protein A - Sepharose beads for another 45 minutes. After washing, 50 μl of the SDS gel loading buffer was added to the bead and boiled for 2 minutes. Autoradiographies of the metabolically labeled proteins precipitated by antibody binding were performed as above.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Characterization of autoantibodies

Autoantibodies were detected against several parathyroid specific proteins in 17 AH sera that we tested by immunoblotting as described above. Twelve (70%) reacted to a protein of 70 kDa, and 16 (94%) to one of 80 kDa. The 70 and 80 kDa proteins were localized to the cytosolic fraction of the parathyroid extracts. Four of 20 sera (20%) so tested had autoantibodies which reacted with a doublet 120-140 kDa protein in the membrane fraction. Sera from 50 patients with other autoimmune diseases as well as 22 normal controls were also tested, and none of them reacted with any of the above specific parathyroid proteins (Table 2):

Table 2. Autoantibody Reactivity To Human Parathyroid Gland (Immunoblot)

Disease	Cytosolic		Membrane
	70 kDa	80 kDa	120-140 kDa
Hypoparathyroidism	12/17 (70%)	16/17 (94%)	4/20 (20%)
Addison disease	0/10	0/10	0/10
Graves disease	0/10	0/10	0/10
Hashimoto thyroiditis	0/12	0/12	0/12
IDD	0/10	0/10	0/10
Vitiligo	0/8	0/8	0/8
Normal Control	0/22	0/22	0/22

The autoantibodies were also detectable using a normal dog parathyroid gland as antigen source in immunoblotting. However, they could not be detected using cultured human melanocytes or rat pituitary cells as antigen sources.

Example 2 – Identification of the autoantigens

Since the parathyroid 120-140 antigen has the same molecular weight as the CA-SR dependent upon its degree of glycosylation, we tested the possibility that the receptor itself was the autoantigen by three different experimental approaches.

In the first approach, the reactivities of rabbit anti-CA-SR IgG and patient sera were tested by immunoblotting using parathyroid gland membranes as the antigen source. The patient sera reacted to a 120-140 kDa protein, which closely matched that recognized by the rabbit anti-CA-SR IgG. Normal control sera and the same concentration of IgG from pre-immunized rabbit did not so react.

In the second approach, the reactivity of rabbit anti-CA-SR IgG and patient sera was examined using transfected HEK-293 cells as an antigen source in immunoblotting experiments. This cell line had been transfected with a human CA-SR cDNA. The patient sera again reacted

to a 120-140 kDa protein, which closely matched that recognized by the anti-CA-SR IgG raised in rabbits (Fig.1). Six AH patient sera (30%) reacted to the CA-SR from this source, but none of the 15 normal control sera did so. In addition, the 6 positive sera did not react to non-transfected or wild type HEK-293 cells which did not express CA-SR proteins (Table 3):

Table 3. Autoantibody Reactivity To Recombinant Ca^{2+}-Sensing Receptor (Immunoblot)

Ag source	AH patients	Normal	Rabbit anti-CaR	Rabbit control
Transfected HEK-293 cell	6/20 (30%)	0/15	+	-
Wild type HEK-293 cell	0/6	0/10	-	-

In the third approach, the localization of the antigenic epitopes was determined by immunoprecipitation of *in vitro* translated CA-SR antigen in lysate from a rabbit reticulocyte expression system. The patient sera reacted to both 70 kDa and 60 kDa proteins of the extracellular domain of the molecule, whereas no normal control sera did so (Fig 2). The 60 kDa is the non-glycosylated form according to the size of the cDNA insert (1.6 kb) while the 70 kDa is the fully glycosylated form resulting from the addition of canine pancreatic microsome membranes.

The cytosolic domain was translated as a 60 kDa protein and no glycosylation occurred after exposure to the microsomal membranes as expected. None of the patient sera reacted with the cytosolic or intracellular domain of the CA-SR (data not shown). In summary, 14 (56%) of AH patient sera reacted to the extracellular domain of the recombinantly expressed CA-SR, whereas none of the 25 AH patient sera reacted to the intracellular domain of the molecule. The autoantibody frequencies might have been higher if newly diagnosed patients had been exclusively studied. None of the 22 normal control sera reacted to either domain of the CA-SR. (Table 4):

Table 4. Autoantibody Reactivity To In Vitro Translated Domains Of Calcium Sensing Receptor (Immunoprecipitation)

Ag source	AH patient	Normal
Ca^{2+}-R Extracellular	14/25 (56%)	0/22
Ca^{2+}-R Intracellular	0 /25	0/22

Example 3 – Isolation, Cloning and Expression of the 70 and 80 kilodalton autoantigens:

The purified or cloned, expressed and purified 70 kilodalton or 80 kilodalton cytosolic antigens present in parathyroid cells are obtained by preparing an affinity column. This is achieved by immobilizing antiserum from an AH patient known to react with these cytosolic proteins. Activated SEPHAROSE beads for this purpose are obtained from Pharmacia and reacted with the serum in a ratio of 1 gram beads to 10 mg serum. A second column is prepared in the same way, except that serum from an AH negative patient is used.

A cytosolic extract of parathyroid is prepared by homogenizing parathyroid gland, preferably fresh from a morgue, in a hypotonic solution containing a cocktail of protease inhibitors. The membranes and debris are removed by centrifugation at a minimum of 25,000 rpm for an hour, and the cytosolic supernatant decanted. All proteins other than the 70 kDa or 80 kDa proteins are adsorbed out of the cytosolic parathyroid preparation by passage of the extract over the column prepared with immobilized AH negative serum. The flow-through of this first column is then passed over the immobilized AH serum column, and the bound, purified antigens eluted by reducing the pH. The affinity column eluate is then concentrated and subjected to size-exclusion HPLC to separate the 70 kDa from the 80 kDa antigen. The resulting isolated antigens may be used directly in the diagnostic method of this invention.

Alternatively, the proteins may be subjected to amino acid analysis and the amino acid sequence is used to design oligonucleotide probes for cloning the genes encoding these proteins. A human parathyroid cDNA library is screened and positive clones isolated. The clones are sequenced to confirm that they contain sequence encoding the 70 or 80 kDa proteins, and positive clones are subcloned such that the antigen coding sequence is placed in an expression vector, which is then transfected into mammalian cells and grown in culture.

Once expressed, the recombinant proteins are recovered and isolated in essentially the same manner described above for the isolation of the naturally occurring antigens.

Example 4 – Assay for Detecting AH or propensity for AH and other Autoimmune Diseases:

CA-SR rich membranes and 70 and 80 kDa parathyroid antigens are contacted with serum from a patient suspected of having autoimmune hypoparathyroidism or for whom it is important to rule out this possible condition.

The presence of AH specific autoantibodies is detected by contacting the patients' serum with biotin labeled AH antigen (70 kDa protein, 80 kDa protein, CA-SR membranes, or antigenic fragments thereof). The reaction is then contacted with a bead coated with immobilized avidin which reacts with the biotin on the AH antigen. If any antigen-antibody complex is formed, this is detected by contacting the beads with anti-human antiserum having a detectable label, such as a radioactive, fluorescent, enzyme or other moiety.

Example 5 – Therapeutic Method

In addition to providing the detection methods described above, our identification of the AH antigens provides a method for antigen mediated immunotherapy based upon the use of recombinant or purified protein antigen as a therapeutic agent to restore immune tolerance in AH. Thus, a method of treating patients with autoimmune hypoparathyroidism or those at risk of developing autoimmune hypoparathyroidism comprises:

(a) detecting the disease or propensity of developing the disease by screening the patient's serum for autoantibody to a 120-140 kDa antigen, a 70 kDa antigen, an 80 kDa antigen, or a combination of these antigen;

(b) treating patients positive in the test of step (a) either with

(i) compensatory PTH therapy,

(ii) antibodies raised against antibodies that recognize the antigens used in step (a),

(iii) antigens of step (a) to restore immune tolerance to AH associated antigens, or

(iv) a combination of treatments (i) - (iii).

The antigens are preferably provided in relatively small doses to avoid induction of anaphylaxis. The mode of administration may be by intravenous administration of the autoantigens or antigenic fragments or peptide thereof, to induce anergy of auto-reactive elements. The dosages and regimen of repeating such treatment is defined by routine clinical studies. In addition, the proteins and peptides may be used for subcutaneous immunizations to induce a switch from Th2 to Th1 responses or formation of blocking antibodies. Yet another alternative is oral administration to induce anergy and immunosuppressive effects on by-stander autoimmune responses.

In a preferred method of the subject invention, prevention or treatment involves the administration of autoantigens to the susceptible individual. AH, as disclosed herein, has an autoimmune etiopathogenesis. Various mechanisms have been proposed that would account for the beneficial value of administering autoantigens as a preventive treatment. In addition, it is also well known in the art that the administration of autoantigens can be used to induce immunological non-responsiveness, that is, specific tolerance of the antigen. See U.S. Patent No. 5,114, 844; Nagler-Anderson *et al.*. (1986) *Proc. Natl. Acad. Sci USA* 83:7443-7446; Miller *et al.*. (1984) *Clin. Immunol. Immunopathol.* 31:231-240; Silverman *et al.*. (1983) *J. Immunol.* 131:2651-2661; Michael (1989) *Immune Invest.* 18:1049-1054. The administration of the AH auto-antigens according to the subject invention can be done using procedures, formulations, and administration routes well known in the art. As one skilled in the art having the benefit of this disclosure would appreciate, the administration of the AH auto-antigens or peptides can be by, for example, parenteral, oral, intranasal, or by modification of the patient's genome to express an antigenic epitope.

Example 6 – Kits for Assay of Autoantibodies Associated with Acquired Hypoparathyroidism

A reagent kit can be provided which facilitates convenient analysis of sera samples using the antigens of the subject invention. Kits can be prepared which utilize recombinant or synthetically produced peptides to serve as an antigen for the autoantibodies. The principles and methods for ELISA and RIA technologies to detect antibodies are well-established.

As an example, for the ELISA assay, one such kit could comprise the following components:

1. One or more of the AH antigens of the subject invention;
2. Enzyme (e.g., peroxidase);
3. Conjugated animal anti-human immunoglobulin; and
4. Positive and negative controls.

The above kit could be modified to include 96 well plastic plates, colorimetric reagents, ELISA readers, blocking reagents, and wash buffers.

Also by way of example, for the RIA, one such kit could comprise the following components:

1. One or more of the AH antigens of the subject invention;
2. Wash buffers;
3. Polyethylene glycol;
4. Goat or sheep antihuman precipitating (second) antibodies; and
5. Positive and negative controls.

Either of the above kits may be modified to include any appropriate laboratory supplies.

In addition to the use of immunoprecipitation techniques, the subject invention can be practiced utilizing any other procedures which facilitate detecting the presence of AH auto-antibodies. For example, other immunological methods which can be used include enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). The principles and experimental methods of these procedures are well known to those skilled in the art. The assays can be carried out rapidly and efficiently by the use of the natural or recombinant proteins which bind with the AH auto-antibodies. Both whole cell and cell lysate procedures are familiar to those working in this field and can be readily employed to detect the AH antibodies.

The amino acid sequence of the AH auto-antigens identified herein can be analyzed to ascertain immunologically reactive epitopes. These epitopes are amino acid sequences which will react immunologically with the auto-antibodies. These sequences can then be produced recombinantly. For recombinant production, the DNA coding for the epitopes is inserted into a vector which is then used to transform an appropriate host cell to express the desired amino acid sequence. Although bacteria, insects, yeasts, and mammalian cells could all serve as

appropriate hosts, if protein folding is an important factor in the reactivity of the epitope, then an eukaryotic cell would be a preferred host.

Purified protein or lysate of the cells producing the protein could be used for the assays.

Also, an alternative to using the AH auto-antigens would be to use antibodies generated to the AH auto-antigens, otherwise known as an anti-antibody. This antibody would immunoprecipitate with the AH auto-antigens, and the detection could be carried out as described above.

It should be understood that the examples and embodiment described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

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Claims

1. A method for detecting acquired hypoparathyroidism (AH) in a person, or propensity for developing autoimmune hypoparathyroidism and the propensity to develop other autoimmune diseases which are commonly associated with development of autoimmune hypoparathyroidism, which comprises contacting a sample of serum from said person with an antigen associated with AH selected from the human calcium sensing receptor, or antigenic subportions thereof, and parathyroid specific cytosolic antigens which are associated with autoimmune hypoparathyroidism, and detecting binding of antibodies present in said serum sample with said antigen, wherein the presence of said binding indicates that said person may have or may be at risk for developing AH.

2. The method of claim 1 wherein the antigenic subportion of said human calcium sensing receptor is the external domain of said receptor.

3. The method of claim 1 which comprises detecting the presence of serum antibodies which react with a 70 kilodalton cytosolic antigen present in parathyroid cells.

4. The method of claim 1 which comprises detecting the presence of serum antibodies which react with an 80 kilodalton cytosolic antigen present in parathyroid cells.

5. A method for obtaining isolated 70 kilodalton parathyroid cytosolic antigen, or antigenic sub-fragments thereof, which reacts with antibodies associated with acquired hypoparathyroidism, which comprises immunoaffinity chromatography using antiserum which binds to said antigen.

6. A method for obtaining isolated 80 kilodalton parathyroid cytosolic antigen, or antigenic sub-fragments thereof, which reacts with antibodies associated with acquired hypoparathyroidism, which comprises immunoaffinity chromatography using antiserum which binds to said antigen.

7. A method for obtaining isolated polynucleotide which encodes the antigen of claim 5, which comprises sequencing said antigen to obtain at least partial amino acid sequence and then screening a genomic or cDNA library with an oligonucleotide which encodes said amino acid sequence.

8. A method for obtaining isolated polynucleotide which encodes the antigen of claim 6, which comprises sequencing said antigen to obtain at least partial amino acid sequence and then screening a genomic or cDNA library with an oligonucleotide which encodes said amino acid sequence.

9. A recombinant cell transfected with the polynucleotide obtained according to the method of claim 7.

10. A recombinant cell transfected with the polynucleotide obtained according to the method of claim 8.

11. An antigen expressed from the polynucleotide obtained according to the method of claim 7.

12. An antigen expressed from the polynucleotide obtained according to the method of claim 8.

13. An antibody to the antigen of claim 11.

14. An antibody to the antigen of claim 12.

15. A method of treating patients with acquired hypoparathyroidism or those at risk of developing acquired hypoparathyroidism which comprises:

(a) detecting the disease or propensity of developing the disease by screening the patient's serum for autoantibody to a 120-140 kDa antigen, a 70 kDa antigen, an 80 kDa antigen, or a combination of these antigen;

(b) treating patients positive in the test of step (a) either with

(i) compensatory PTH therapy,

(ii) antibodies raised against antibodies that recognize the antigens used in step (a),

(iii) antigens of step (a) to restore immune tolerance to AH associated antigens,

or (iv) a combination of treatments (i) - (iii).

16. The method of claim 15 which comprises intravenous, subcutaneous or oral administration of the calcium sensing receptor, extracellular fragments thereof, the 70 kDa parathyroid cytosolic protein recognized by AH anti-serum, the 80 kDa parathyroid cytosolic protein recognized by AH anti-serum, or combinations or antigenic portions thereof.

17. A kit for use in a method for detecting AH in a person, said kit comprising:

(a) the CA-SR or antigenic subportions thereof, the 70 kDa parathyroid cytosolic antigen recognized by AH positive sera, the 80 kDa parathyroid cytosolic antigen recognized by AH positive sera;

(b) animal anti-human immunoglobulin.

18. An isolated antigen having a molecular mass of about 70 kilodaltons, derived from the parathyroid, and having the properties of the antigen isolated according to claim 5.

19. An isolated antigen having a molecular mass of about 80 kilodaltons, derived from the parathyroid and having the properties of the antigen isolated according to claim 6.

Fig. 1

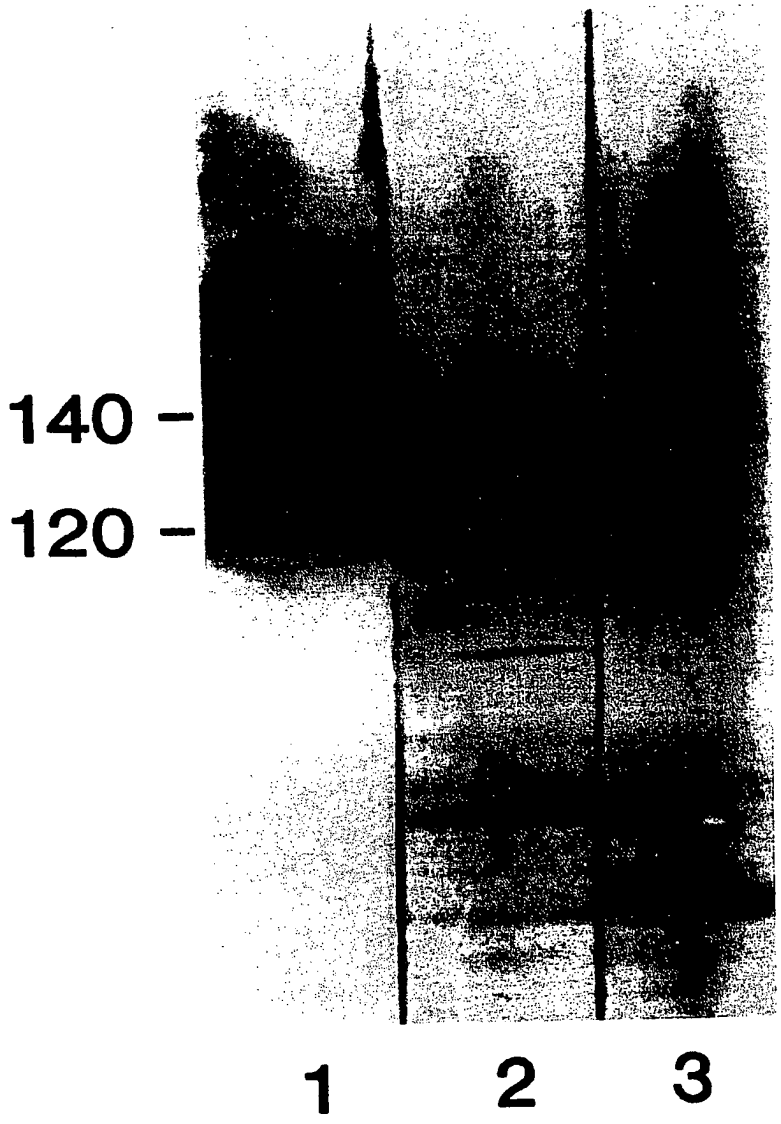
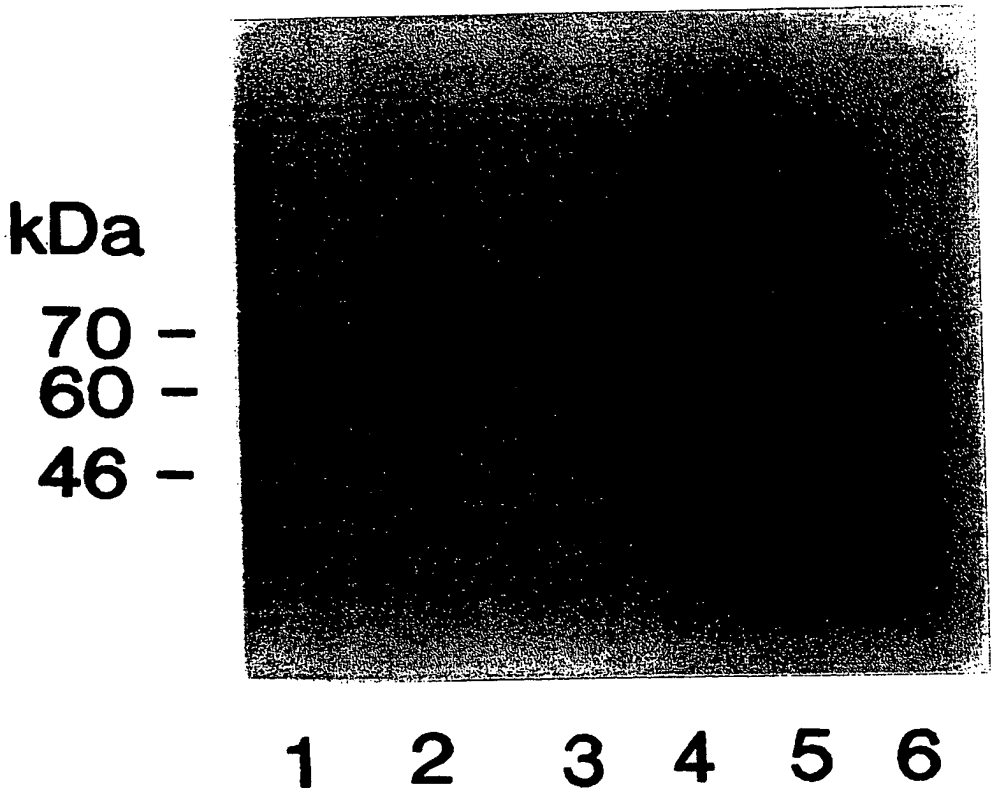


Fig. 2



INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/14032

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 C07K14/705 C07K16/18 C12N15/12 G01N33/53
C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Abstract N. or2-3 77th annual meeting of the endocrine society Washington D.C. 14-17/06/95 XP002019373 cited in the application "Acquired hypoparathyroidism is associated with autoantibodies 70 and 80 kda parathyroid ..." see abstract ---	5,6, 11-14, 18,19
Y	WO,A,94 25604 (R S R LIMITED) 10 November 1994 see page 1, paragraph 1 - paragraph 2; claims 1-46 see page 9, line 1 - page 10, line 18 --- -/--	1-4, 7-10,17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 1996	11. 12. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CLINICAL AND EXPERIMENTAL IMMUNOLOGY, 62 (2). 1985. 353-360., XP000610406 BETTERLE C ET AL: "DEMONSTRATION AND CHARACTERIZATION OF ANTI-HUMAN MITOCHONDRIA AUTOANTIBODIES IN IDIOPATHIC HYPOPARATHYROIDISM AND IN OTHER CONDITIONS" cited in the application see the whole document	1,17
A	WO,A,94 15214 (HENNING BERLIN GMBH ;BERGMANN ANDREAS (DE); MIKLUS METOD (DE); THO) 7 July 1994 see page 5, paragraph 2; claims 1,13	1,17
A	WO,A,94 28019 (AAKERSTROEM GOERAN ;KLARESKOG LARS (SE); JUHLIN CLAES (SE); RASK L) 8 December 1994 cited in the application see claims 1-6	1,17
P,X	J CLIN INVEST, FEB 15 1996, 97 (4) P910-4, UNITED STATES, XP000610031 LI Y ET AL: "Autoantibodies to the extracellular domain of the calcium sensing receptor in patients with acquired hypoparathyroidism [see comments]" see the whole document	1,17

INTERNATIONAL SEARCH REPORT

I rnational application No.
PCT/US 96/ 14032

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 15,16
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15,16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/14032

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9425604	10-11-94	AU-A-	6661094	21-11-94
WO-A-9415214	07-07-94	DE-C-	4243375	01-06-94
		AU-B-	666429	08-02-96
		AU-A-	5810294	19-07-94
		CA-A-	2130486	07-07-94
		EP-A-	0627081	07-12-94
		FI-A-	943816	19-08-94
		JP-T-	7507143	03-08-95
WO-A-9428019	08-12-94	SE-C-	504108	11-11-96
		AU-A-	6902194	20-12-94
		CA-A-	2163013	08-12-94
		EP-A-	0700401	13-03-96
		SE-A-	9301764	25-11-94

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(60) Parent Applications or Grants
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(54) Title: A CHROMOSOME 21 - DERIVED NUCLEIC ACID ENCODING A NOVEL PROTEIN, COMPOSITIONS AND METHOD USING SAME

(57) Abstract

The present invention provides isolated nucleic acids encoding human EHOC-17 protein and isolated EHOC-17 proteins encoded thereby. Further provided are vectors containing invention nucleic acids, probes that hybridize thereto, host cells transformed therewith, antisense oligonucleotides thereto and compositions containing antibodies that specifically bind to invention polypeptides, as well as transgenic non-human mammals that express the invention protein.

A CHROMOSOME 21 - DERIVED NUCLEIC ACID ENCODING A NOVEL PROTEIN, COMPOSITIONS AND METHOD USING SAME

BACKGROUND OF THE INVENTION

A major endeavor in molecular genetics has been made in generating maps of the human genome. Human genome mapping consists, generally, of ordering genomic DNA fragments on their chromosomes using several methods, such as fluorescent *in situ* hybridization (FISH), somatic cell hybrid analysis or random clone fingerprinting. DNA fragments that correspond to marked polymorphic sites can be ordered by genetic linkage analysis. Distances between polymorphic loci are estimated by meiotic recombination frequencies. High resolution maps based upon the estimated distances, however, cannot be constructed easily using such methods because the resolution is low at the molecular level and recombination frequency is not linearly correlated with physical distance.

Thus, various obstacles such as, for example, the difficulty in obtaining highly informative markers and the paucity of identified markers that are evenly spaced along the chromosome are significant weaknesses of the currently available genetic maps. Most of the mapped markers are restriction fragment length polymorphisms (RFLPs) assayed by DNA hybridization. Although maps based on these markers have contributed greatly to the primary mapping of a number of diseases, they are still insufficient for many applications such as mapping rare monofactorial diseases, refining linkage intervals to distances suited for gene identification, and mapping of loci contributing to complex traits.

Genetic linkage mapping is an important technology applied to the study of human biology and, in particular, for the delineation of the molecular basis of disease. Indeed, one of the most commonly used strategies

for studying human inherited diseases is by cloning the responsible gene based on chromosomal location. Genetic linkage maps, therefore, facilitate the identification and mapping of genes involved in monogenic diseases, genes involved in multifactorial disorders, and are useful in carrier detection and prenatal diagnosis of hereditary disorders. A detailed linkage map is also a prerequisite for clone-based physical mapping and DNA sequencing of the entire chromosome.

Human chromosome 21 is a paradigm for large-scale human genome mapping efforts. The smallest human chromosome, chromosome 21 has approximately 50 megabases (Mb) of DNA. Less than 1% of the 2000 genes estimated to be on chromosome 21 are known. A high resolution map of chromosome is of particular interest because of its apparent role in familial Alzheimer disease (FAD), Down's syndrome (DS), amyotrophic lateral sclerosis (ALS), and Finnish progressive myoclonus epilepsy (PME). A gene defect responsible for FAD has been localized to chromosome 21 on the basis of genetic linkage to three pericentromeric loci. The gene encoding the precursor of the Alzheimer-associated amyloid ß protein (APP), the principle component of the senile plaques and cerebrovascular amyloid deposits of Alzheimer disease (AD), has also been mapped to chromosome 21.

The process of developing such a long-range contig map involves the identification and localization of landmarks in cloned genetic fragments. When there are enough landmarks for the size of the cloned fragments, contigs are formed, and the landmarks are simultaneously ordered. Currently, YACs, or yeast artificial chromosomes, are utilized for most mapping of the human genome. YACs permit cloning of fragments of ≥ about 500 Kb. However, some difficulties have been encountered with the manipulation of YAC libraries. For example, in various YAC

libraries, a fraction of the clones result from co-cloning events, i.e., they include in a single clone noncontiguous DNA fragments. A high percentage of YAC clones, particularly clones having high molecular weight inserts, are chimeric. Chimeric clones map to multiple sites on the chromosome and, thus, hamper the progress of mapping and analysis. Another problem endemic to YAC cloning is caused by DNA segments that are unclonable or unstable and tend to rearrange and delete.

Bacteria Artificial Chromosomes (BACs), provide an alternative to the YAC system. BACs mitigate the most problematic aspects of YACs such as, for example the high rate of chimerism and clonal instability. BACs are based on the *E. coli* single-copy plasmid F factor and are capable of faithful propagation of DNA fragments greater than about 300 Kb in size. BACs have a number of physical properties that make them amenable to physical mapping, including easy manipulation and an absence of chimerism. The lack of chimerism and the capacity to propagate large exogenous insert DNAs make the BACs excellent candidates for chromosome walking and the generation of contiguous physical maps.

The need for molecular description of chromosome 21 derives directly from the association with several human genetic diseases. A map of contiguous units (contigs) covering this chromosome will speed the identification of the cause of these diseases. Indeed, a detailed map would provide immediate access to the genomic segment, including any pathological locus, as soon as it has been localized by genetic linkage or cytogenetic analysis.

Thus, a need exists for identifying, characterizing, and mapping the numerous genes that occupy loci on chromosome 21, which will expedite the rapid translation of high resolution chromosome maps into

biological, medical and diagnostic applications. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides isolated nucleic acids encoding human EHOC-17 protein and isolated proteins encoded thereby. Further provided are vectors containing invention nucleic acids, probes that hybridize thereto, host cells transformed therewith, antisense oligonucleotides thereto and compositions containing, antibodies that specifically bind to invention polypeptides and compositions containing, as well as transgenic non-human mammals that express the invention protein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a physical map for the consensus region for HPE1.

Figure 2 shows a physical map for the consensus region for EPM1 in relation to the consensus region for HPE1. The locations of YAC clones, BAC clones and EHOC-1 were indicated by thick bars.

Figure 3 shows the genomic location of EHOC-17 in a physical map for the consensus region for EPM1.

DETAILED DESCRIPTION OF THE INVENTION

Progressive myoclonus epilepsies (PMEs) are a heterogenous group of diseases which are characterized by myoclonus, epileptic seizures and progressive neurological deterioration including ataxia and dementia, Berkovic et al., *New Engl. J. Med.* 315:296-305 (1986). PME of Unverricht-Lundborg type (EPM1) is an autosomal recessive

disorder with frequent consanguinity in Finland and Mediterranean regions with the incidence of at least 1: 20,000 in Finland. Genetic linkage analysis revealed that the locus for EPM1 is on chromosome 21q22.3, Malafosse et al., *Lancet* 339:1080-1081 (1992) and excluded, Lafora disease from this region which is also a member of PME, Lehesjoki et al., *Neurology* 42:1545-1550 (1992). Linkage disequilibrium analysis made it possible to narrow down the candidate region to 300kb spanning the loci of PFKL, D21S25 and D21S154, Lehesjoki et al., *Hum. Mol. Genet.* 2:1229-1234 (1993); Lehesjoki et al., *Human Genetics* 93:668-674 (1994).

Autoimmune polyglandular disease type I (APECED) was also mapped to chromosome 21q22.3 by linkage disequilibrium analysis, Aaltonen, J., et al., *Nature Genet.* 8:83-87 (1994). APECED is an autosomal recessive disease resulting in a variable combination of failure of the parathyroid glands, adrenal cortex, gonads pancreatic ß cells, thyroid gland and gastric parietal cells. Additional affects of APECED include alopecia, vitiligo, hepatitis, chronic mucocutaneous candidiasis, dystrophy of the dental enamel and nails and keratopathy. APECED usually manifests itself in childhood, but tissue specific symptoms may appear throughout adulthood. The APECED locus maps within 500 kb of D21S49 and D21S171.

Holoprosencephaly (HPE) is characterized by impaired cleavage of the embryonic forebrain and incomplete mid-facial development that manifest as a wide range of midfacial anomalies including cyclopia, ethmocephaly cebocephaly, premaxillary agenesis, hypotelorism, and a single maxillary central incisor. The most commonly associated chromosomal abnormality includes dup(3p), del(7q), deletions of chromosome 13, trisomy 13, trisomy 18, and triploidy (Munke, AM J Med Genet 34:237-245 (1989)). The etiology is heterogeneous and may include aneuploidies for chromosomes 2, 3, 7, 13, 18 and 21. In

order to narrow down the candidate region for HPE1, the deletion of 21(q22.3) was characterized in two HP patients by fluorescence *in situ* hybridization and quantitative Southern blot dosage analysis. For the smaller deletion, the regions for D21S25, D21S154, D21S171 and D21S44 were deleted and for D21S42 and D21S49 were not. Combining these data with previous reports of deletion of 21q22.3 (D21S112-ter) without the holoprosencephaly phenotype indicate that the region responsible for holoprosencephaly spans the 1-2Mb region including PFKL and ITGB2 (CD18). Four cases of holoprosencephaly with chromosome 21 anomalies have been published. Estabrooks et al. describe a minute deletion of chromosome 21(q22.3) (Estabrooks et al., AM J Med Genet, 36:306-309 (1990)) suggesting this region as a locus for holoprosencephaly (HPE1).

Described in the instant specification is the construction of the BAC (Bacterial Artificial Chromosome) Shizuya et al., *Proc. Natl. Acad. Sci. USA* 89:8794-8797 (1992) contig of this EPM1-APECED-HPE1 candidate region and the isolation of a novel gene from this contiguous map unit using a direct cDNA selection technique. The BAC contig of this EPM1 region has been reported (Yamakawa et al., Hum. Molec. Genet., 4:709-716 (1995).

In order to isolate genes responsible for these diseases, a cDNA library from a 14-week trisomy 21 fetal brain was constructed using Uni-Zap XR (Stratagene, La Jolla, CA). More than 95% of the clones have inserts ranging from 1-4kb (avg. 2kb). In addition, a direct cDNA selection method was applied to BACs (Bacterial Artificial Chromosomes) in the 21q22.3 region.

Sau3AI linkers were attached to cDNA that was synthesized from a trisomy 21 fetal brain. After digestion with Sau3AI, a second pair of linkers were attached to the cDNA which was then hybridized to biotinylated BAC DNAs

which covered the candidate region. cDNA/BAC DNA hybrid molecules were captured on streptavidin coated magnetic beads, non-specific cDNAs were washed out, and specifically hybridized cDNAs were eluted and subsequently amplified by PCR. Twice selected PCR products were subcloned and analyzed. Southern blot analysis revealed that 21 out of 30 (70%) of the fragments yielded unique bands of the original BACs. Using these fragments as probes, a cDNA was isolated from the library. The approximately 3.1 kb cDNA subclone (EHOC-17) maps proximal to neighboring D21S25 and exhibited approximately 48% homology to the yeast PWP2 gene. The loci of this gene maps within the consensus region where holoprosencephaly, EPM1 and APECED are localized. DNA sequence analysis of the 3.1 kb cDNA showed a complete coding sequence of 2759 bp (nucleotides 25-2784 of SEQ ID NO:1) which revealed amino acid sequence homology with yeast PWP2 protein.

Five BAC clones were isolated from the total human genomic DNA BAC library (Shizuya et al., *Proc. Natl. Acad. Sci. USA* 89:8794-8797 (1992)) by PCR screening using sequence tagged sites (STSs) containing PFKL, D21S25, D21S154 and CD18. Physical maps of the HPE1-EPM1-APECED consensus region with these BAC clones and YAC clones (Chumakov et al., *Nature* 359:380-387 (1992)) are shown in Figures 1 and 2. BAC-1 (230kb) and BAC-2 (210kb) were positive for D21S25. BAC-3 (170kb) was positive for D21S25 and PFKL. Agarose gel electrophoresis of EcoRI-digested BAC DNAs and Southern blot analysis showed that these 3 BACs are overlapping. BAC-4 was identical to BAC-3. BAC-5 (100kb) was positive for CD18.

Direct cDNA selection was performed on 5 BAC DNAs (four of which were overlapping) which span the consensus region. EcoRI digestion of subclone DNAs revealed that 10% of the clones were chimeric. The average size of the inserts of non-chimeric clones was 400bp. Forty non-

chimeric subclones of selected cDNAs were analyzed by using EcoRI-digested BAC DNA Southern blots. Twenty-eight clones (70%) showed unique signals on the BAC blots, 6 clones (15%) showed repetitive, and 6 clones (15%) did not show any signal on these blots. Using insert DNAs of these subclones as probes, a trisomy 21 fetal brain cDNA library was screened. A cDNA, encoding a novel protein, containing poly $(A)^+$ tails was isolated and designated EHOC-17. The EHOC-17 cDNA subclone was used for Southern blot analysis using EcoRI-digested BAC DNA blots. BAC-1 and BAC-2 showed unique multiple band signals indicating that the cDNA originated from BAC-1 and BAC-2.

Northern blot analysis using the insert of EHOC-17 cDNA revealed at least one transcript expressed ubiquitously in multiple adult tissues (e.g., heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, and the like). Fluorescence *in-situ* hybridization is also performed on lymphocytes from a normal individual using the insert from the EHOC-17 cDNA subclone as a probe. Discrete signals are seen on chromosome 21q22.3 confirming the loci.

The complete sequence of the cDNA encoding EHOC-17 revealed an open reading frame of 2759 bp (nucleotides 25-2784 of SEQ ID NO:1). The initiator ATG was located within a good Kozak consensus sequence Kozak, M., *J. Mol. Biol.* 196:947-950 (1987); Kozak, M., *Nuc. Acid Res.* 15:8125-8148 (1987). A homology search of the amino acid sequence of this open reading frame (ORF) with genes registered in Genbank/EMBL showed that this gene product is related to yeast PWP2 protein.

Accordingly, the present invention provides isolated nucleic acids, which encode a novel EHOC-17 protein, wherein such nucleic acids are derived from human chromosome 21, specifically at the q23.2 locus, which is the site of mutation(s) that cause PME, HPE1, and APECED.

The term "nucleic acids" (also referred to as polynucleotides) encompasses RNA as well as single and double-stranded DNA and cDNA. As used herein, the phrase "isolated" means a nucleic acid that is in a form that does not occur in nature. One means of isolating a nucleic acid encoding an EHOC-17 polypeptide is to probe a mammalian genomic library with a natural or artificially designed DNA probe using methods well known in the art. DNA probes derived from the EHOC-17 gene are particularly useful for this purpose. DNA and cDNA molecules that encode EHOC-17 polypeptides can be used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian (e.g., mouse, rat, rabbit, pig, and the like), or other animal sources, or to isolate related cDNA or genomic clones by the screening of cDNA or genomic libraries, by methods described in more detail below. Examples of nucleic acids are RNA, cDNA, or isolated genomic DNA encoding an EHOC-17 polypeptide. Such nucleic acids may have coding sequences substantially the same as the coding sequence shown in SEQ ID NO:1, or at least nucleotides 25-2784 of SEQ ID NO:1.

As used herein, "mammalian" refers to the variety of species from which the invention EHOC-17 protein is derived, e.g., human, rat, mouse, rabbit, monkey, baboon, bovine, porcine, ovine, canine, feline, and the like. A preferred EHOC-17 protein herein, is human EHOC-17.

In one embodiment of the present invention, cDNAs encoding the invention EHOC-17 proteins disclosed herein include substantially the same nucleotide sequence as set forth in SEQ ID NO:1. Preferred cDNA molecules encoding the invention proteins include the same nucleotide sequence as nucleotides 25-2784 of SEQ ID NO:1.

As employed herein, the term "substantially the same nucleotide sequence" refers to DNA having sufficient identity to the reference polynucleotide, such that it will

hybridize to the reference nucleotide under moderately stringent hybridization conditions. In one embodiment, DNA having substantially the same nucleotide sequence as the reference nucleotide sequence encodes substantially the same amino acid sequence as that set forth in SEQ ID NO:2, or a larger amino acid sequence including SEQ ID NO:2. In another embodiment, DNA having "substantially the same nucleotide sequence" as the reference nucleotide sequence has at least 60% identity with respect to the reference nucleotide sequence. DNA having at least 70%, more preferably at least 90%, yet more preferably at least 95%, identity to the reference nucleotide sequence is preferred.

This invention also encompasses nucleic acids which differ from the nucleic acids shown in SEQ ID NO:1, but which have the same phenotype, i.e., those that encode all or a fragment of a protein that is substantially the same amino acid sequence set forth in SEQ ID NO:2. Phenotypically similar nucleic acids are also referred to as "functionally equivalent nucleic acids". As used herein, the phrase "functionally equivalent nucleic acids" encompasses nucleic acids characterized by slight and non-consequential sequence variations that will function in substantially the same manner to produce the same protein product(s) as the nucleic acids disclosed herein. In particular, functionally equivalent nucleic acids encode polypeptides that are the same as those disclosed herein or that have conservative amino acid variations. For example, conservative variations include substitution of a non-polar residue with another non-polar residue, or substitution of a charged residue with a similarly charged residue. These variations include those recognized by skilled artisans as those that do not substantially alter the tertiary structure of the protein.

Further provided are nucleic acids encoding EHOC-17 polypeptides that, by virtue of the degeneracy of the

genetic code, do not necessarily hybridize to the invention nucleic acids under specified hybridization conditions. Preferred nucleic acids encoding the invention polypeptide are comprised of nucleotides that encode substantially the same amino acid sequence set forth in SEQ ID NO:2.

Thus, an exemplary nucleic acid encoding an invention EHOC-17 polypeptide may be selected from:

(a) DNA encoding the amino acid sequence set forth in SEQ ID NO:2,

(b) DNA that hybridizes to the DNA of (a) under moderately stringent conditions, wherein said DNA encodes biologically active EHOC-17, or

(c) DNA degenerate with respect to either (a) or (b) above, wherein said DNA encodes biologically active EHOC-17.

Hybridization refers to the binding of complementary strands of nucleic acid (i.e., sense:antisense strands or probe:target-DNA) to each other through hydrogen bonds, similar to the bonds that naturally occur in chromosomal DNA. Stringency levels used to hybridize a given probe with target-DNA can be readily varied by those of skill in the art.

Stringency of hybridization, as used herein, refers to conditions under which polynucleotide hybrids are stable. As known to those of skill in the art, the stability of hybrids is a function of sodium ion concentration and temperature (See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual 2d Ed. (Cold Spring Harbor Laboratory, 1989; incorporated herein by reference). Stringency levels used to hybridize a given probe with target-DNA can be readily varied by those of skill in the art.

As used herein, the phrase "moderately stringent" hybridization refers to conditions that permit target-DNA to bind a complementary nucleic acid that has about 60%, preferably about 75%, more preferably about 85%, homology to the target DNA; with greater than about 90% homology to target-DNA being especially preferred. Preferably, moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 65°C. Denhart's solution and SSPE (see, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989) are well known to those of skill in the art as are other suitable hybridization buffers.

The phrase "high stringency hybridization" refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C (i.e., if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.1X SSPE, and 0.1% SDS at 65°C.

The phrase "low stringency hybridization" refers to conditions equivalent to hybridization in 10% formamide, 5X Denhart's solution, 6X SSPE, 0.2% SDS at 42°C, followed by washing in 1X SSPE, 0.2% SDS, at 50°C. Denhart's solution and SSPE (see, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989) are well known to those of skill in the art as are other suitable hybridization buffers.

As used herein, the term "degenerate" refers to codons that differ in at least one nucleotide from a

reference nucleic acid, e.g., SEQ ID NO:1, but encode the same amino acids as the reference nucleic acid. For example, codons specified by the triplets "UCU", "UCC", "UCA", and "UCG" are degenerate with respect to each other since all four of these codons encode the amino acid serine.

Preferred nucleic acids encoding the invention polypeptide(s) hybridize under moderately stringent, preferably high stringency, conditions to substantially the entire sequence, or substantial portions (i.e., typically at least 15-30 nucleotides) of the nucleic acid sequence set forth in SEQ ID NO:1.

The invention nucleic acids can be produced by a variety of methods well-known in the art, e.g., the methods described herein, employing PCR amplification using oligonucleotide primers from various regions of SEQ ID NO:1, and the like.

Also provided are isolated peptides, polypeptides(s) and/or protein(s) encoded by the invention nucleic acids which are EHOC-17 polypeptides. The EHOC-17 polypeptide comprises a protein of approximately 919 amino acids in length. The complete amino acid sequence encoding the human EHOC-17 polypeptide is set forth in SEQ ID NO:2.

As used herein, the term "isolated" means a protein molecule free of cellular components and/or contaminants normally associated with a native *in vivo* environment. Invention polypeptides and/or proteins include any isolated natural occurring allelic variant, as well as recombinant forms thereof. The EHOC-17 polypeptides can be isolated using various methods well known to a person of skill in the art. The methods available for the isolation and purification of invention proteins include, precipitation, gel filtration, ion-

exchange, reverse-phase and affinity chromatography. Other well-known methods are described in Deutscher et al., Guide to Protein Purification: Methods in Enzymology Vol. 182, (Academic Press, 1990), which is incorporated herein by reference. Alternatively, the isolated polypeptides of the present invention can be obtained using well-known recombinant methods as described, for example, in Sambrook et al., *supra*., 1989).

An example of the means for preparing the invention polypeptide(s) is to express nucleic acids encoding the EHOC-17 in a suitable host cell, such as a bacterial cell, a yeast cell, an amphibian cell (i.e., oocyte), or a mammalian cell, using methods well known in the art, and recovering the expressed polypeptide, again using well-known methods. Invention polypeptides can be isolated directly from cells that have been transformed with expression vectors, described below in more detail. The invention polypeptide, biologically active fragments, and functional equivalents thereof can also be produced by chemical synthesis. As used herein, "biologically active fragment" refers to any portion of the polypeptide represented by the amino acid sequence in SEQ ID NO:2 that can assemble into a cationic channel permeable to Ca^{2+} which is activated by acetylcholine. Synthetic polypeptides can be produced using Applied Biosystems, Inc. Model 430A or 431A automatic peptide synthesizer (Foster City, CA) employing the chemistry provided by the manufacturer.

As used herein, the phrase "EHOC-17" refers to substantially pure native EHOC-17 protein, or recombinantly expressed/produced (i.e., isolated or substantially pure) proteins, including variants thereof encoded by mRNA generated by alternative splicing of a primary transcript, and further including fragments thereof which retain native biological activity. Preferred invention polypeptides are comprised of substantially the same amino acid sequence set

forth in SEQ ID NO:2. As used herein, the phrase "functional polypeptide" means a EHOC-17 that can produce an anti-EHOC-17 antibody that binds to the amino acid sequence set forth in SEQ ID NO:2.

Modification of the invention nucleic acids, polypeptides or proteins with the following phrases: "recombinantly expressed/produced", "isolated", or "substantially pure", encompasses nucleic acids, peptides, polypeptides or proteins that have been produced in such form by the hand of man, and are thus separated from their native *in vivo* cellular environment. As a result of this human intervention, the recombinant nucleic acids, polypeptides and proteins of the invention are useful in ways that the corresponding naturally occurring molecules are not, such as identification of selective drugs or compounds.

Sequences having "substantially the same sequence" homology are intended to refer to nucleotide sequences that share at least about 75%, preferably about 80%, yet more preferably about 90% identity with invention nucleic acids; and amino acid sequences that typically share at least about 75%, preferably about 85%, yet more preferably about 95% amino acid identity with invention polypeptides. It is recognized, however, that polypeptides or nucleic acids containing less than the above-described levels of homology arising as splice variants or that are modified by conservative amino acid substitutions, or by substitution of degenerate codons are also encompassed within the scope of the present invention.

The present invention provides the isolated polynucleotide operatively linked to a promoter of RNA transcription, as well as other regulatory sequences. As used herein, the phrase "operatively linked" refers to the functional relationship of the polynucleotide with

regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of a polynucleotide to a promoter refers to the physical and functional relationship between the polynucleotide and the promoter such that transcription of DNA is initiated from the promoter by an RNA polymerase that specifically recognizes and binds to the promoter, and wherein the promoter directs the transcription of RNA from the polynucleotide.

Promoter regions include specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. Additionally, promoter regions include sequences that modulate the recognition, binding and transcription initiation activity of RNA polymerase. Such sequences may be *cis* acting or may be responsive to *trans* acting factors. Depending upon the nature of the regulation, promoters may be constitutive or regulated. Examples of promoters are SP6, T4, T7, SV40 early promoter, cytomegalovirus (CMV) promoter, mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, and the like.

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding

sites can be inserted immediately 5' of the start codon to enhance expression. (See, for example, Kozak, J. Biol. Chem. 266:19867 (1991)). Similarly, alternative codons, encoding the same amino acid, can be substituted for coding sequences of the EHOC-17 polypeptide in order to enhance transcription (e.g., the codon preference of the host cell can be adopted, the presence of G-C rich domains can be reduced, and the like).

Also provided are vectors comprising invention nucleic acids. Examples of vectors are viruses, such as baculoviruses and retroviruses, bacteriophages, cosmids, plasmids and other recombination vehicles typically used in the art. Polynucleotides are inserted into vector genomes using methods well known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of restricted polynucleotide. These synthetic linkers contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are well known and available in the art.

Further provided are vectors comprising nucleic acids encoding EHOC-17 polypeptides, adapted for expression in a bacterial cell, a yeast cell, an amphibian cell (i.e., oocyte), a mammalian cell and other animal cells. The vectors additionally comprise the regulatory elements necessary for expression of the nucleic acid in the bacterial, yeast, amphibian, mammalian or animal cells so located relative to the nucleic acid encoding EHOC-17 polypeptide as to permit expression thereof.

As used herein, "expression" refers to the process by which nucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the nucleic acid is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eucaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al. *supra*). Similarly, a eucaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the invention polypeptide.

The present invention provides transformed host cells that recombinantly express EHOC-17 polypeptides. An example of a transformed host cell is a mammalian cell comprising a plasmid adapted for expression in a mammalian cell. The plasmid contains nucleic acid encoding an EHOC-

17 polypeptide and the regulatory elements necessary for expression of invention proteins. Various mammalian cells may be utilized as hosts, including, for example, mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Ltk- cells, etc. Expression plasmids such as those described *supra* can be used to transfect mammalian cells by methods well known in the art such as, for example, calcium phosphate precipitation, DEAE-dextran, electroporation, microinjection or lipofection.

The present invention provides nucleic acid probes comprising nucleotide sequences capable of specifically hybridizing with sequences included within nucleic acids encoding EHOC-17 polypeptides, for example, a coding sequence included within the nucleotide sequence shown in SEQ ID NO:1. As used herein, a "probe" is a single-stranded DNA or RNA that has a sequence of nucleotides that includes at least 15 contiguous bases set forth in SEQ ID NO:1. Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences within the ORF, and the like. Full-length or fragments of cDNA clones can also be used as probes for the detection and isolation of related genes. When fragments are used as probes, preferably the cDNA sequences will be from the carboxyl end-encoding portion of the cDNA, and most preferably will include predicted transmembrane domain-encoding portions of the cDNA sequence. Transmembrane domain regions can be predicted based on hydropathy analysis of the deduced amino acid sequence using, for example, the method of Kyte and Doolittle, J. Mol. Biol. 157:105 (1982).

As used herein, the phrase "specifically hybridizing" encompasses the ability of a polynucleotide to recognize a sequence of nucleic acids that are complementary thereto and to form double-helical segments via hydrogen bonding between complementary base pairs.

Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable agent, such as a radioisotope, a fluorescent dye, and the like, to facilitate detection of the probe. Invention probes are useful to detect the presence of nucleic acids encoding the EHOC-17 polypeptide. For example, the probes can be used for *in situ* hybridizations in order to locate biological tissues in which the invention gene is expressed. Additionally, synthesized oligonucleotides complementary to the nucleic acids of a nucleotide sequence encoding EHOC-17 polypeptide are useful as probes for detecting the invention genes, their associated mRNA, or for the isolation of related genes using homology screening of genomic or cDNA libraries, or by using amplification techniques well known to one of skill in the art.

Also provided are antisense oligonucleotides having a sequence capable of binding specifically with any portion of an mRNA that encodes EHOC-17 polypeptides so as to prevent translation of the mRNA. The antisense oligonucleotide may have a sequence capable of binding specifically with any portion of the sequence of the cDNA encoding EHOC-17 polypeptides. As used herein, the phrase "binding specifically" encompasses the ability of a nucleic acid sequence to recognize a complementary nucleic acid sequence and to form double-helical segments therewith via the formation of hydrogen bonds between the complementary base pairs. An example of an antisense oligonucleotide is an antisense oligonucleotide comprising chemical analogs of nucleotides.

Compositions comprising an amount of the antisense oligonucleotide, described above, effective to reduce expression of EHOC-17 polypeptides by passing through a cell membrane and binding specifically with mRNA

encoding EHOC-17 polypeptides so as to prevent translation and an acceptable hydrophobic carrier capable of passing through a cell membrane are also provided herein. The acceptable hydrophobic carrier capable of passing through cell membranes may also comprise a structure which binds to a receptor specific for a selected cell type and is thereby taken up by cells of the selected cell type. The structure may be part of a protein known to bind to a cell-type specific receptor.

Antisense oligonucleotide compositions are useful to inhibit translation of mRNA encoding invention polypeptides. Synthetic oligonucleotides, or other antisense chemical structures are designed to bind to mRNA encoding EHOC-17 polypeptides and inhibit translation of mRNA and are useful as compositions to inhibit expression of EHOC-17 associated genes in a tissue sample or in a subject.

In accordance with another embodiment of the invention, kits for detecting mutations and aneuploidies in chromosome 21 at locus q22.3 comprising at least one invention probe or antisense nucleotide.

The present invention provides means to modulate levels of expression of EHOC-17 polypeptides by employing synthetic antisense oligonucleotide compositions (hereinafter SAOC) which inhibit translation of mRNA encoding these polypeptides. Synthetic oligonucleotides, or other antisense chemical structures designed to recognize and selectively bind to mRNA, are constructed to be complementary to portions of the nucleotide sequences shown in SEQ ID NO:1. The SAOC is designed to be stable in the blood stream for administration to a subject by injection, or in laboratory cell culture conditions. The SAOC is designed to be capable of passing through the cell membrane in order to enter the cytoplasm of the cell by

virtue of physical and chemical properties of the SAOC which render it capable of passing through cell membranes, for example, by designing small, hydrophobic SAOC chemical structures, or by virtue of specific transport systems in the cell which recognize and transport the SAOC into the cell. In addition, the SAOC can be designed for administration only to certain selected cell populations by targeting the SAOC to be recognized by specific cellular uptake mechanisms which bind and take up the SAOC only within select cell populations. For example, the SAOC may be designed to bind to a receptor found only in a certain cell type, as discussed *supra*. The SAOC is also designed to recognize and selectively bind to target mRNA sequence, which may correspond to a sequence contained within the sequence shown in SEQ ID NO:1. The SAOC is designed to inactivate target mRNA sequence by either binding thereto and inducing degradation of the mRNA by, for example, RNase I digestion, or inhibiting translation of mRNA target sequence by interfering with the binding of translation-regulating factors or ribosomes, or inclusion of other chemical structures, such as ribozyme sequences or reactive chemical groups which either degrade or chemically modify the target mRNA. SAOCs have been shown to be capable of such properties when directed against mRNA targets (see Cohen et al., TIPS, 10:435 (1989) and Weintraub, Sci. American, January (1990), pp.40; both incorporated herein by reference).

This invention also provides compositions containing an acceptable carrier and any of an isolated, purified EHOC-17 polypeptide, an active fragment thereof, or a purified, mature protein and active fragments thereof, alone or in combination with each other. These polypeptides or proteins can be recombinantly derived, chemically synthesized or purified from native sources. As used herein, the term "acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as phosphate

buffered saline solution, water and emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents.

Further provided are antibodies having specific reactivity with EHOC-17 polypeptides of the present invention. Active fragments of antibodies are encompassed within the definition of "antibody".

Invention antibodies can be produced by methods known in the art using invention polypeptides, proteins or portions thereof as antigens. For example, polyclonal and monoclonal antibodies can be produced by methods well known in the art, as described, for example, in Harlow and Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory 1988), which is incorporated herein by reference. Invention polypeptides can be used as immunogens in generating such antibodies. Alternatively, synthetic peptides can be prepared (using commercially available synthesizers) and used as immunogens. Amino acid sequences can be analyzed by methods well known in the art to determine whether they encode hydrophobic or hydrophilic domains of the corresponding polypeptide. Altered antibodies such as chimeric, humanized, CDR-grafted or bifunctional antibodies can also be produced by methods well known in the art. Such antibodies can also be produced by hybridoma, chemical synthesis or recombinant methods described, for example, in Sambrook et al., *supra.*, and Harlow and Lane, *supra*. Both anti-peptide and anti-fusion protein antibodies can be used. (see, for example, Bahouth et al., Trends Pharmacol. Sci. 12:338 (1991); Ausubel et al., Current Protocols in Molecular Biology (John Wiley and Sons, NY 1989) which are incorporated herein by reference).

Invention antibodies also can be used to isolate invention polypeptides. Additionally the antibodies are

useful for detecting the presence of invention polypeptides, as well as analysis of chromosome localization, and structural as well as functional domains. Methods for detecting the presence of EHOC-17 polypeptides on the surface of a cell comprise contacting the cell with an antibody that specifically binds to EHOC-17 polypeptides, under conditions permitting binding of the antibody to the polypeptides, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of invention polypeptides on the surface of the cell. With respect to the detection of such polypeptides, the antibodies can be used for *in vitro* diagnostic or *in vivo* imaging methods.

Immunological procedures useful for *in vitro* detection of target EHOC-17 polypeptides in a sample include immunoassays that employ a detectable antibody. Such immunoassays include, for example, ELISA, Pandex microfluorimetric assay, agglutination assays, flow cytometry, serum diagnostic assays and immunohistochemical staining procedures which are well known in the art. An antibody can be made detectable by various means well known in the art. For example, a detectable marker can be directly or indirectly attached to the antibody. Useful markers include, for example, radionucleotides, enzymes, fluorogens, chromogens and chemiluminescent labels.

Further, invention antibodies can be used to modulate the activity of the EHOC-17 polypeptide in living animals, in humans, or in biological tissues or fluids isolated therefrom. Accordingly, compositions comprising a carrier and an amount of an antibody having specificity for EHOC-17 polypeptides effective to block binding of naturally occurring ligands to invention polypeptides. A monoclonal antibody directed to an epitope of EHOC-17 polypeptide molecules present on the surface of a cell and having an amino acid sequence substantially the same as an

amino acid sequence for a cell surface epitope of an EHOC-17 polypeptide shown in SEQ ID NO:2, can be useful for this purpose.

The present invention further provides transgenic non-human mammals that are capable of expressing nucleic acids encoding EHOC-17 polypeptides. Also provided are transgenic non-human mammals capable of expressing nucleic acids encoding EHOC-17 polypeptides so mutated as to be incapable of normal activity, i.e., do not express native EHOC-17. The present invention also provides transgenic non-human mammals having a genome comprising antisense nucleic acids complementary to nucleic acids encoding EHOC-17 polypeptides so placed as to be transcribed into antisense mRNA complementary to mRNA encoding EHOC-17 polypeptides, which hybridizes thereto and, thereby, reduces the translation thereof. The nucleic acid may additionally comprise an inducible promoter and/or tissue specific regulatory elements, so that expression can be induced, or restricted to specific cell types. Examples of nucleic acids are DNA or cDNA having a coding sequence substantially the same as the coding sequence shown in SEQ ID NO:1. An example of a non-human transgenic mammal is a transgenic mouse. Examples of tissue specificity-determining elements are the metallothionein promoter and the L7 promoter.

Animal model systems which elucidate the physiological and behavioral roles of EHOC-17 polypeptides are produced by creating transgenic animals in which the expression of the EHOC-17 polypeptide is altered using a variety of techniques. Examples of such techniques include the insertion of normal or mutant versions of nucleic acids encoding an EHOC-17 polypeptide by microinjection, retroviral infection or other means well known to those skilled in the art, into appropriate fertilized embryos to produce a transgenic animal. (See, for example, Hogan et

al., Manipulating the Mouse Embryo: A Laboratory Manual (Cold Spring Harbor Laboratory, 1986). Another technique, homologous recombination of mutant or normal versions of these genes with the native gene locus in transgenic animals, may be used to alter the regulation of expression or the structure of EHOC-17 polypeptides (see, Capecchi et al., Science 244:1288 (1989); Zimmer et al., Nature 338:150 (1989); which are incorporated herein by reference). Homologous recombination techniques are well known in the art. Homologous recombination replaces the native (endogenous) gene with a recombinant or mutated gene to produce an animal that cannot express native (endogenous) protein but can express, for example, a mutated protein which results in altered expression of EHOC-17 polypeptides. In contrast to homologous recombination, microinjection adds genes to the host genome, without removing host genes. Microinjection can produce a transgenic animal that is capable of expressing both endogenous and exogenous EHOC-17 protein. Inducible promoters can be linked to the coding region of nucleic acids to provide a means to regulate expression of the transgene. Tissue specific regulatory elements can be linked to the coding region to permit tissue-specific expression of the transgene. Transgenic animal model systems are useful for *in vivo* screening of compounds for identification of specific ligands, i.e., agonists and antagonists, which activate or inhibit protein responses.

Invention nucleic acids, oligonucleotides (including antisense), vectors containing same, transformed host cells, polypeptides and combinations thereof, as well as antibodies of the present invention, can be used to screen compounds *in vitro* to determine whether a compound functions as a potential agonist or antagonist to invention polypeptides. These *in vitro* screening assays provide information regarding the function and activity of invention polypeptides, which can lead to the

identification and design of compounds that are capable of specific interaction with one or more types of polypeptides, peptides or proteins.

In accordance with still another embodiment of the present invention, there is provided a method for identifying compounds which bind to EHOC-17 polypeptides. The invention proteins may be employed in a competitive binding assay. Such an assay can accommodate the rapid screening of a large number of compounds to determine which compounds, if any, are capable of binding to EHOC-17 proteins. Subsequently, more detailed assays can be carried out with those compounds found to bind, to further determine whether such compounds act as modulators, agonists or antagonists of invention proteins.

In another embodiment of the invention, there is provided a bioassay for identifying compounds which modulate the activity of invention polypeptides. According to this method, invention polypeptides are contacted with an "unknown" or test substance (in the presence of a reporter gene construct when antagonist activity is tested), the activity of the polypeptide is monitored subsequent to the contact with the "unknown" or test substance, and those substances which cause the reporter gene construct to be expressed are identified as functional ligands for EHOC-17 polypeptides.

In accordance with another embodiment of the present invention, transformed host cells that recombinantly express invention polypeptides can be contacted with a test compound, and the modulating effect(s) thereof can then be evaluated by comparing the EHOC-17-mediated response (via reporter gene expression) in the presence and absence of test compound, or by comparing the response of test cells or control cells (i.e., cells

that do not express EHOC-17 polypeptides), to the presence of the compound.

As used herein, a compound or a signal that "modulates the activity" of invention polypeptides refers to a compound or a signal that alters the activity of EHOC-17 polypeptides so that the activity of the invention polypeptide is different in the presence of the compound or signal than in the absence of the compound or signal. In particular, such compounds or signals include agonists and antagonists. An agonist encompasses a compound or a signal that activates EHOC-17 protein expression. Alternatively, an antagonist includes a compound or signal that interferes with EHOC-17 protein expression. Typically, the effect of an antagonist is observed as a blocking of agonist-induced protein activation. Antagonists include competitive and non-competitive antagonists. A competitive antagonist (or competitive blocker) interacts with or near the site specific for agonist binding. A non-competitive antagonist or blocker inactivates the function of the polypeptide by interacting with a site other than the agonist interaction site.

As understood by those of skill in the art, assay methods for identifying compounds that modulate EHOC-17 activity generally require comparison to a control. One type of a "control" is a cell or culture that is treated substantially the same as the test cell or test culture exposed to the compound, with the distinction that the "control" cell or culture is not exposed to the compound. For example, in methods that use voltage clamp electrophysiological procedures, the same cell can be tested in the presence or absence of compound, by merely changing the external solution bathing the cell. Another type of "control" cell or culture may be a cell or culture that is identical to the transfected cells, with the exception that the "control" cell or culture do not express

native proteins. Accordingly, the response of the transfected cell to compound is compared to the response (or lack thereof) of the "control" cell or culture to the same compound under the same reaction conditions.

In yet another embodiment of the present invention, the activation of EHOC-17 polypeptides can be modulated by contacting the polypeptides with an effective amount of at least one compound identified by the above-described bioassays.

The invention will now be described in greater detail with reference to the following non-limiting examples.

EXAMPLE 1
Construction of BAC Contig

BAC library construction of total human genomic DNA was performed as described in Shizuya et al., *Proc. Natl. Acad. Sci. USA* 89:8794-8797 (1992). BAC clones were screened by PCR using STSs (PFKL, D21S25, D21S154, CD18). The loci of these BAC clones were confirmed by fluorescence *in-situ* hybridization. Insert size of BAC clones was measured by running pulsed-field gel electrophoresis after digesting DNA with NotI.

EXAMPLE 2
Direct cDNA Selection

Direct selection procedures were similar to those described in Morgan et al., *Nucleic Acid Res.* 20:5173-5179 (1992) with some modifications. Total RNA was isolated from 14 week trisomy 21 fetal brain using TRI region™ (Molecular Research Center, Inc.). Poly (A)$^+$ RNA was isolated using Poly (A) Quick® mRNA isolation kit

(STRATAGENE). Double stranded cDNA was synthesized using SuperScript™ Choice System (GIBCO BRL) from 5μg trisomy 21 fetal brain poly $(A)^+$ RNA using 1μg oligo $(dT)_{15}$ or 0.1μg random hexamer. The entire synthesis reaction was purified by Gene Clean®II kit (BIO101, Inc.) and then kinased. Sau3AI linker was attached to the cDNA which was subsequently digested with Sau3AI. The reaction was purified using Gene Clean. MboI linker was attached to the cDNA and the reaction purified by Gene Clean (Morgan et al., *Nucleic Acid Res.* 20:5173-5179 (1992)). The synthesized product was amplified by PCR using one strand of MboI linker (5'CCTGATGCTCGAGTGAATTC3') (SEQ ID NO:3) as a primer. PCR cycling conditions were 40 cycles of 94°C/15 seconds, 60°C/23 seconds, 72°C/2 minutes in a 100μl of 1x PCR buffer (Promega), 3mM $MgCl_2$, 5.0 units of Taq polymerase (Promega), 2μM primer and 0.2mM dNTPs.

Five BAC DNAs (total 2.5μg) were prepared using QIAGEN plasmid kit and were biotinylated using Nick Translation Kit and biotin-16-dUTP (Boehringer Manneheim). 3μg of heat denatured PCR amplified cDNA was annealed with 3μg of heat denatured COT1 DNA (BRL) in 100μl hybridization buffer (750mM NaCl, 50mM $NaPO_4$(pH7.2), 5mM EDTA, 5x Denhardt's, 0.05% SDS and 50% formamide) at 42°C for two hours. After prehybridization, 1.2μg of heat denatured biotinylated BAC DNA was added and incubated at 42°C for 16 hours. cDNA-BAC DNA hybrids were precipitated with EtOH and dissolved in 60μl of 10mM Tris-HCl (pH 8.0), 1mM EDTA. After addition of 40μl 5M NaCl, the DNA was incubated with magnetic beads (Dynabeads M-280, Dynal) at 25°C for 1 hour with gentle rotating to allow attachment of the DNA to the magnetic beads. The beads were then washed twice by pipetting in 400μl of 2x SSC, setting in magnet holder (MPC-E_{TM}, Dynal) for 30 seconds and removing the supernatant. Four additional washes were performed in 0.2x SSC at 68°C for 10 minutes each with transfer of the beads to new tubes at each wash. cDNAs were eluted in 100μl of

distilled water for 10 minutes at 80°C with occasional mixing. The eluted cDNAs were amplified by PCR as described above. After twice repeating the selection procedure using magnetic beads, amplified cDNAs were digested with EcoRI and subcloned into pBluescript II.

EXAMPLE 3

Southern blot analysis

Gel electrophoresis of DNA was carried out on 0.8% agarose gels in 1x TBE. Transfer of nucleic acids to Nybond N+ nylon membrane (Amersham) was performed according to the manufacturer's instruction. Probes were labelled using RadPrime Labeling System (BRL). Hybridization was carried out at 42°C for 16 hours in 50% formamide, 5x SSPE, 5x Denhardt's 0.1% SDS, 100μg/ml denatured salmon sperm DNA. The filters were washed once in 1x SSC, 0.1% SDS at room temperature for 20 minutes, and twice in 0.1x SSC, 0.1% SDS for 20 minutes at 65°C. The blots were exposed onto X-ray film (Kodak, X-OMAT-AR).

EXAMPLE 4

cDNA Library Screening

A trisomy 21 fetal brain cDNA library was constructed using ZAP-cDNA® synthesis kit (STRATAGENE) which generates a unidirectional cDNA library. Briefly, double-stranded cDNA was synthesized from 5μg trisomy 21 fetal brain poly(A)$^+$ RNA using a hybrid oligo(dT)-XhoI linker primer with 5-methyl dCTP. An EcoRI linker was attached to the cDNA which was subsequently digested with EcoRI and XhoI, and then cloned into UNI-ZAP XR vector. The library was packaged using Gigapack® II Gold packaging extract. The titer of the original library was 1.1×10^6 p.f.u./package. The library was amplified once. A blue-white color assay indicated that 99% of the clones had inserts. The average

size of the inserts was 1.9 kb, as calculated from 14 clones.

Screening of the trisomy 21 fetal brain cDNA library was performed using selected cDNA fragments. Phages were plated to an average density of 1×10^5 per 175 cm^2 plate. Plaque lifts of 20 plates (2×10^6 phages) were made using duplicated nylon membranes (Hybond-N+; Amersham). Hybridized membranes were washed to final stringency of 0.2x SSC, 0.1x SDS at 65°C. The filters were exposed overnight onto X-ray film. Phages were subcloned into the plasmid vector pBluescript II SK(-) by M13-mediated excision for further analysis.

EXAMPLE 5
Northern Blot Analysis

cDNA inserts were excised from the vector by digestion with XhoI and EcoRI. After labeling using the random priming method, the fragments were used a probes for Northern hybridization using Multiple Tissue Northern Blot (Clontech).

EXAMPLE 6
Metaphase Preparation

Chromosomes were prepared using a BrdU block, (Zabel et al. in *Proc. Natl. Acad. Sci. USA* 80:6932-6936 (1983)) with some modification. Briefly, human peripheral lymphocytes were grown for 72 hours at 37°C in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with L-glutamine (2mM), 15% fetal calf serum, penicillin (100 IU/ml), streptomycin (0.05mg/ml) and 0.02% phytohemagglutinin. The cells were blocked in S-phase by adding 5-bromo-deoxyuridine (0.8mg/ml) for 16 hours. Cells were washed once with HBSS (Hanks Balanced Salt Solution) (GIBCO BRL, Gaithersburg, MD) to remove the synchronizing agent and

were released by incubating for five to six more hours in medium supplemented with 2.5μg/ml thymidine. Cultures were harvested by the addition of 0.1μg/ml colcemid for 10 minutes followed by 0.075 M KCl hypotonic solution for 15 minutes at 37°C prior to fixation with a 3:1 mixture of methanol and acetic acid, for 1-5 minutes.

EXAMPLE 7

High Quality Chromosome Preparation

To obtain high quality chromosome preparations, the metaphase spreads were prepared by letting one drop of suspension fall onto alcohol-cleaned slides, evenly and flat, completely free of cytoplasm. The slides were then placed above a container filled with heated water for 20-60 seconds depending on the ambient humidity. The optimum conditions were determined by first checking several slides under a phase contrast microscope. For best results, the slides should be aged at room temperature for at least 2-3 weeks prior to *in situ* hybridization. Slides are best stored at -70°C after aging. The day before denaturation, slides are removed from -70°C and kept overnight at 4°C. Slides are then left at room temperature for 1-2 hours before baking them at 55-60°C for 2 hours, followed by denaturation in 70% formamide (at 66-70°C). For best results, fresh slides are denatured at 66°C.

EXAMPLE 8

Probe Fragment Size for Mapping Small DNA Probes

The fragment size of a probe labeled by nick translation is preferably around 100-200 bp and the concentration of probe DNA in the hybridization mixture is preferably in the range of 20-40 ng/μl (i.e., 200-400 ng/slide). This concentration can increase non-specific binding, but generally will produce good signal/noise ratio. For reducing non-specific background hybridization

of repeat sequences, a small amount of CotI DNA can be used with cDNA probes (i.e., 1-3μg of CotI with 200-400 ng of probe DNA).

EXAMPLE 9

Biotinylation of Probes

DNA probes were labeled with biotin-14-dATP by nick translation (GIBCO BRL). Unincorporated nucleotides were separated by chromatography (Sephadex G-50). The mean fragment size of all DNA probes was set at around 200 bp, with a range of 100-600 bp, established by nondenaturing agarose gels.

EXAMPLE 10

In situ Hybridization

To obtain specific hybridization of single-copy sequences with small DNA probes, the FISH method described by Lichter et al. in *Science* 247:64-69 (1990) was modified. RNase treatment is unnecessary for slides made more than one year prior to their use. When necessary, RNase treatment consisted of 100μg/ml RNase, placed on slides for 20 minutes at 37°C, followed by dehydration through a cold ethanol series of 70%, 90% and 100%. The denaturation of slides was performed at 67-70°C in 70% formamide/2x SSC (0.15 M NaCl, 0.015 M Sodium Citrate) for 1-2 minutes (fresh chromosome preparations require a shorter time). The hybridization buffer (10μl) containing the biotinylated probes together with 3μg Cot 1 DNA and 7μg sonicated salmon sperm DNA, (the ratio of probe: Cot 1 DNA is 1:15-30; total DNA concentration at 1μg/μl) was placed on each slide. A coverslip was then applied and sealed with rubber cement, followed by overnight incubation in a humidified chamber at 37°C.

EXAMPLE 11

Detection of Probe Signals by Immunofluorescence

After overnight hybridization, the slides were washed at 44°C in 50% formamide (v/v)/2X SSC (4 x 5 minutes) and then at 50-60°C in 2X SSC (3 x 5 minutes). The rinsing time was shorter (3 x 2 minutes) for small probes (insert size <1.5 Kb). To increase the intensity of the fluorescence signal produced by smaller probes, it is necessary to reduce the stringency of post-hybridization washes (2X SSC, at 45-50°C). Slides were then incubated for 10-20 minutes at 37°C with 100µl of 4X SSC containing 3% bovine serum albumin (BSA) and 0.1% Tween 20 for blocking. The buffer was then replaced with FITC-Avidin (0.5µg/ml in 4X SSC/1% BSA/0.1% Tween 20), for 30 minutes at 37°C. After three 5 minutes washes at 45°C in 2X SSC/0.1% Tween 20, the hybridization signal was amplified by the addition of a biotinylated goat anti-avidin antibody layer (5µg/ml) for 30 minutes at 37°C. The slides were preincubated in 5% goat serum/2X SSC/3% BSA/0.1% Tween 20 for 10-20 minutes prior to the amplification step to reduce immunological non-specific binding. After washing, a second layer of FITC-avidin was added to the slides as previously described. To increase the intensity of the hybridization signal, a second round of amplification can be applied as necessary. The slides were then washed in 2X SSC/0.1% Tween 20 three times at 45°C and briefly left to drain.

EXAMPLE 12

Chromosome R-Banding

To view the chromosome bands and fluorescence signals simultaneously, the dyes chromomycin A3 and distamycin A were used as counterstain (Schweizer in *Chromosoma* 58:307-324 (1976); Schweizer in *Hum. Genet* 57:1-14 (1981); and Magenis et al. in *Hum. Genet* 69:300-303

(1985)). Immediately following the last detection, the slides were rinsed briefly in McIlavane's buffer (pH 8.5-9.0) Schweizer et al., *supra*. (diluted 1:1 with distilled water) prior to staining. Subsequently, 100μl of chromomycin A3 (0.5mg/ml in 1/2 McIlavane's buffer pH 8.5-9.0) was placed on slides for 10 minutes - 1 hour at room temperature in the dark. The staining time required depends on the freshness of the slides; fresher slides need longer staining times, whereas aged slides may need only 10 minutes. After a first round of staining, the slides were rinsed for 1 minute in 1/2 McIlavane's buffer and the excess fluid was shaken off. This was followed by a second round of staining by placing 50μl of 0.1mg/ml distamycin A on the slide, incubating for 1-2 minutes at room temperature, followed by rinsing, as above.

If using DNA probes having small inserts (e.g., expected to produce faint signals), at this point, the slides can be mounted with a thin layer of anti-fade solution containing p-phenylenediamine in phosphate buffer (Johnson et al. in *J. Immunol. Methods* 43:349-350 (1981)).

Maximum banding resolution and minimal fading is obtained by two rounds of chromomycin A3 and distamycin A staining. In addition, the best banding results are obtained by staining the slides immediately following the detection step, but no later than 2-3 days. Slides are best viewed through the microscope immediately after staining, but no later than 3 days for small probes (1.0 Kb - 2.5 Kb) or 1-2 weeks for larger probes (>2.5-3 Kb). Only a light counterstain is needed before examining the slide under a fluorescence microscope. In fact, an advantage of this banding technique is that the counterstaining step can be repeated at any point after hybridization to make the chromosomes brighter, if necessary. Further, after image capture, using computerized systems, the banding pattern

may be selectively bleached to more clearly view the FITC hybridization signal.

EXAMPLE 13

Microscopy and Photography

The slides were viewed with a Zeiss Axiophot 100 or Axiovert 135 fluorescence microscope (Zeiss, Inc., Thornwood, NY). The FITC and chromomycin A3 are both excited by using a 400-490nm band pass exciter, 460nm dichroic, and 470nm barrier (Zeiss filter set #05). The hybridized segments appear as bright green-blue or bright yellow-green spots, while the rest of the chromosome bands appear dim green. Kodak Technical pan (ASA100) film was used for black and white photographs. Color images were captured by a cooled-CCD camera (Photometrics CH 250, Photometrics Ltd., Tuscon, AZ) using the BDS (Biological Detection Systems, Pittsburgh, PA) imaging software.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SEQ ID NO:1

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151  AAAAACAACA AATCTGACAC GCTGCCCCTG GCCACTCGGT ACAACGTCAA
201  GTGCGTGGGG CTGTCCCCGG ATGGCCGCCT CGCTATCATC GTCGATGAAG
251  GGGGCGATGC GCTGCTGGTC AGCCTGGTCT GCAGGTCTGT GCTGCACCAC
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351  GAAGTTTGTT GTCACAAAGG GTAACATTGC CCAGATGTAT CATGCCCCTG
401  GGAAGAAGCG GGAGTTCAAC GCCTTCGTTC TGGACAAGAC CTATTTTGGG
451  CCCTACGATG AGACCACCTG CATCGACTGG ACGGATGACT CCAGGTGCTT
501  TGTGGTTGGG AGCAAAGACA TGTCCACCTG GGTGTTCGGA GCCGAGCGCT
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1601  GCCTATGGGA CATGTTTGAC AGCTGGAGGA CCAAGGAGAC GCTGGCCCTG
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1701  TGTGGCCACA CTGAACTCAC AGATCACCTT CTGGGACCCT GAGAACGCGG
1751  TGCAGACGGG CTCCATTGAG GGCAGGCATG ACCTCAAGAC TGGCAGGAAG
1801  GAGCTGGACA AGATTACAGC CAAGCACGCG GCCAAGGGGA AGGCCTTCAC
1851  CGCCCTGTGC TACTCTGCAG ACGGCCACAG CATCCTGGCG GGAGGCATGT
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1951  TTCGAGATCT CTTGCAACCT GTCTCTGGAC GCCATGGAGG AATTTTTGAA
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2151  CTTCTCTCCC ACTGGGCGCT GCTGGGCGGC CACCACCACG GAGGGACTCC
2201  TCATCTACTC CCTGGACACC CGCGTGCTCT TTGACCCGTT TGAGCTGGAC
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2751  AGGCAGAGAC TCAGAAGAAG AGATGCTGGC CTAGAGCCAG CCGGTTGCAG
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2851  ACCTGGGCCA GCAAAGAGGA GGGGTCCAGA GAACAGCTGA AATACTGTCA
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2951  AGCGAGCCGA CGGGTCACGC CAGGGGCCGG CACGCACTGG CACCTGGCCC
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SEQ ID NO:2

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101 SVSFSPDGRK FVVTKGNIAQ MYHAPGKKRE FNAFVLDKTY FGPYDETTCI
151 DNTDDSRCFV VGSKDMSTWV FGAERWDNLI YYALGGIIKD AIVAFFESNS
201 LDLYSLSQDG VLCKVNQCDTP PEGLRLKPPA GWKADLLQRE EEEEEEEDQE
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451 RTFTSPRPTQ FSCVAVDASG EIVSAGAQDS FEIFVWSKQT GRLLDVLSGH
501 EGPISGLCFN PHKSVLASAS WDKTVRLWDM FDSWRTKETL ALTSDALAVT
551 FRPDGAELAV ATLNSQITFW DPENAVQTGS IEGRHDLKTG RKELDKITAK
601 HAAKGKAFTA LCYSADGHSI LAGGMSKFVC IYHYREQILM KRFEISCNLS
651 LDAMEEFLNR RKMTEFGNLA LIDQDAGQED GVAIPLPGVR KGDMSSRHFK
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751 REALRQQDFT RAILMALRLN ESKLVQEALE AVPRGEIEVV TSSLPELYVE
801 KVLEFLASSF EVSRHLEFYL LWTHKLLMLH GQKLKSRAGT LLPVIQFLQK
851 SIQRHLDDLS KLCSMMHYNM QYALAVSKQR GTKRSLDPLG SEEEAEASED
901 DSLHLLGGGG RDSEEEMLA
```

What is claimed is:

1. Isolated nucleic acid encoding a mammalian EHOC-17 polypeptide, or fragment thereof.

2. Isolated nucleic acid according to claim 1, wherein said nucleic acid, or fragments thereof, is selected from:

(a) DNA encoding the amino acid sequence set forth in SEQ ID NO:2, or

(b) DNA that hybridizes to the DNA of (a) under moderately stringent conditions, wherein said DNA encodes biologically active EHOC-17, or

(c) DNA degenerate with respect to either (a) or (b) above, wherein said DNA encodes biologically active EHOC-17.

3. A nucleic acid according to claim 2, wherein said nucleic acid hybridizes under high stringency conditions to the EHOC-17 coding portion of SEQ ID NO:1.

4. A nucleic acid according to claim 2, wherein the nucleotide sequence of said nucleic acid is substantially the same as that set forth in SEQ ID NO:1.

5. A nucleic acid according to claim 4, wherein the nucleotide sequence of said nucleic acid is the same as that set forth in SEQ ID NO:1.

6. A nucleic acid according to claim 2, wherein said nucleic acid is cDNA.

7. A vector containing the nucleic acid of claim 2.

8. Recombinant cells containing the nucleic acid of claim 2.

9. An oligonucleotide comprising at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleic acids of the nucleotide sequence set forth in SEQ ID NO:1.

10. An oligonucleotide according to claim 9, wherein said oligonucleotide is labeled with a detectable marker.

11. An antisense oligonucleotide capable of specifically binding to and modulating the translation of mRNA encoded by said nucleic acid according to claim 2.

12. A kit for detecting mutations, aneuploidies or the presence of EHOC-17 cDNA sequence comprising at least one oligonucleotide according to claim 9.

14. An oligonucleotide composition comprising chemical analogues of the nucleic acid of claim 2 operatively linked to a promoter of RNA transcription.

15. Isolated EHOC-17 polypeptide and functional equivalents thereof.

16. Isolated EHOC-17 polypeptide according to claim 15, wherein said polypeptide has substantially the same amino acid sequence as that set forth in SEQ ID NO:2.

17. Isolated EHOC-17 polypeptide according to claim 16, wherein said polypeptide has the same amino acid sequence as that set forth in SEQ ID NO:2.

18. Isolated EHOC-17 polypeptide according to claim 15, wherein said polypeptide is encoded by a nucleotide sequence that is substantially the same nucleotide sequence as that set forth in SEQ ID NO:1.

19. Isolated EHOC-17 polypeptide according to claim 18, wherein said polypeptide is encoded by the nucleotide sequence set forth in SEQ ID NO:1.

20. Method for expression of a EHOC-17-related protein, said method comprising culturing cells of claim 8 under conditions suitable for expression of said EHOC-17 protein.

21. An isolated anti-EHOC-17 antibody having specific reactivity with an EHOC-17 polypeptide according to claim 15.

22. Antibody according to claim 21, wherein said antibody is a monoclonal antibody.

23. An antibody according to claim 21, wherein said antibody is a polyclonal antibody.

24. A composition comprising an amount of the antisense oligonucleotide according to claim 11 effective to modulate expression of a human EHOC-17 polypeptide and an acceptable hydrophobic carrier capable of passing through a cell membrane.

25. A transgenic nonhuman mammal expressing exogenous nucleic acid encoding a EHOC-17 polypeptide according to claim 15.

26. A transgenic nonhuman mammal according to claim 25, wherein said nucleic acid encoding said EHOC-17 polypeptide has been mutated, and wherein the EHOC-17 polypeptide so expressed is not native EHOC-17.

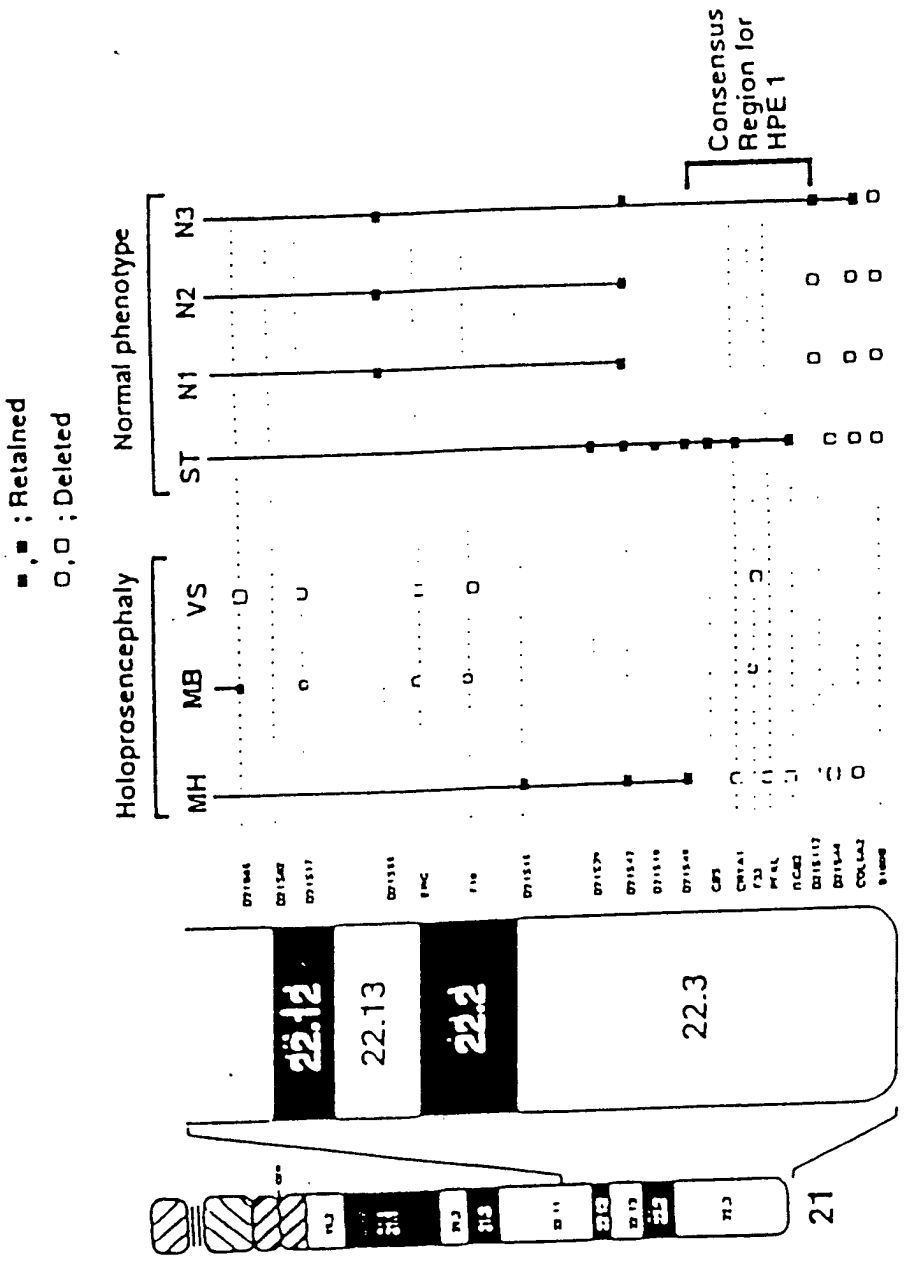
27. A transgenic nonhuman mammal according to claim 25, wherein the transgenic nonhuman mammal is a mouse.

28. A method for identifying nucleic acids encoding a mammalian EHOC-17 polypeptide, said method comprising:

contacting a sample containing nucleic acids with an oligonucleotide according to claim 9, wherein said contacting is effected under high stringency hybridization conditions, and identifying nucleic acids which hybridize thereto.

29. A method for detecting the presence of a human EHOC-17 polypeptide, said method comprising contacting a test sample with an antibody according to claim 21, detecting the presence of an antibody-EHOC-17 complex, and therefor detecting the presence of a human EHOC-17 polypeptide in said test sample.

30. Single strand DNA primers for amplification of EHOC-17 nucleic acid, wherein said primers comprise a nucleic acid sequence derived from the nucleic acid sequence set forth as SEQ ID NO:1.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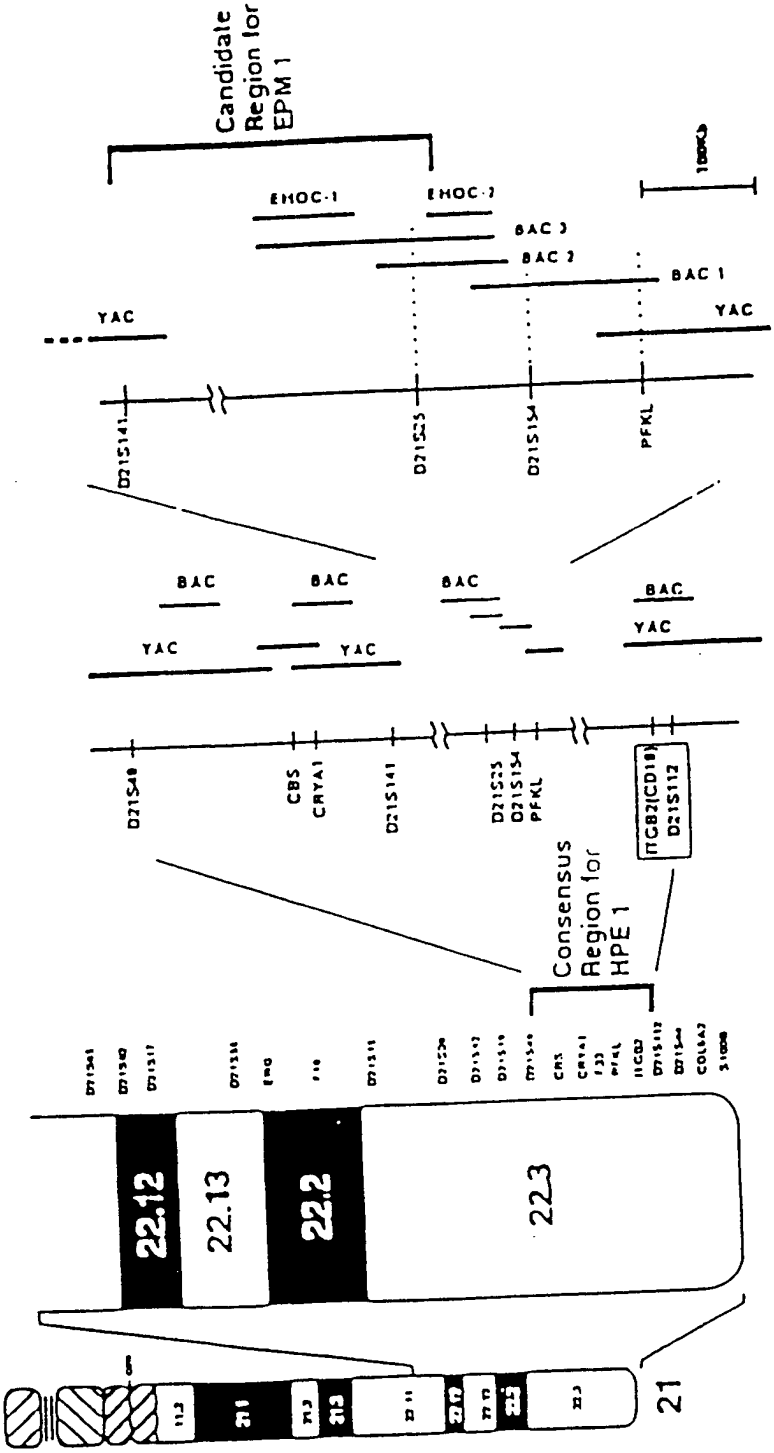
22.2
22.3
21

FIGURE 1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CBS
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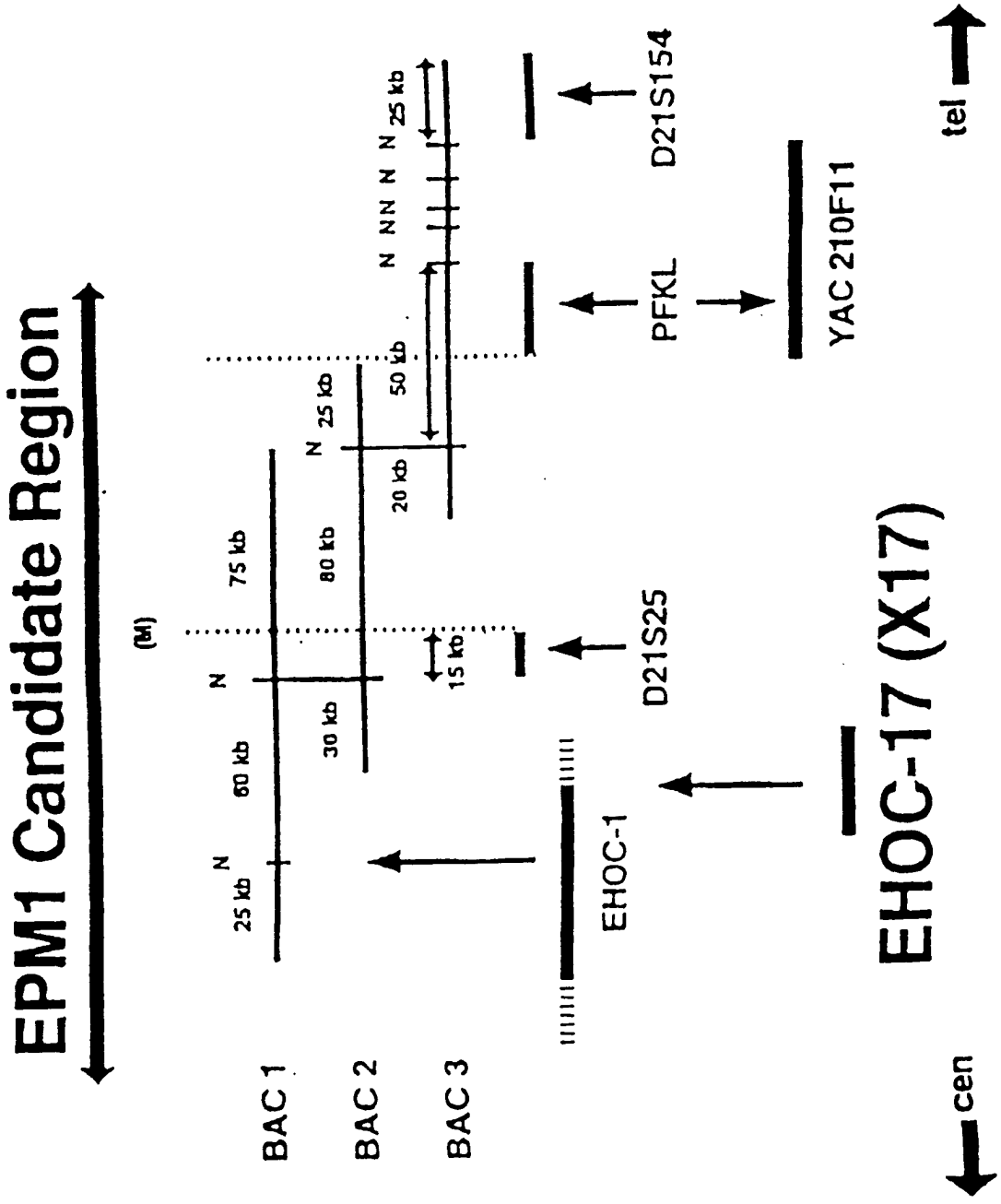
22.13
22.2
22.3
21

FIGURE 2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BAC 2
BAC 3
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80 kb
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25 kb
20 kb
50 kb
N NN N N 25 kb
EHOC-1
D21S25
PFKL
YAC 210F11
D21S154
EHOC-17 (X17)
cen
tel

FIGURE 3